Posttranslational generation of Cα-formylglycin in eukaryotic sulfatases: development of the biochemical approach for the characterisation and purification of the modifying enzyme

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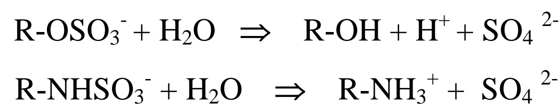
1. Introduction

1.1.The sulfatases

Sulfate esters are participating in a wide spectrum of important biological reactions. They are found in a number of organic compounds of the cell, like glycosaminoglycans (dermatan sulfate, chondroitin sulfate, keratan sulfate, heparan sulfate), sulfated glycolipids (cerebroside sulfate), tyrosine-sulfate carrying proteins and sulfated hydroxysteroids (cholesterol sulfate, dehydroepiandrosterone sulfate). These sulfate esters are hydrolysed by enzymes belonging to the family of sulfate ester sulfohydrolases, or sulfatases, showing high substrate specificity. The sulfatases catalyse the hydrolysis of sulfate esters such as O-sulfates and N-sulfates:



$$R\text{-}OSO_3^- + H_2O \Rightarrow R\text{-}OH + H^+ + SO_4^{2-}$$

$$R\text{-}NHSO_3^- + H_2O \Rightarrow R\text{-}NH_3^+ + SO_4^{2-}$$

1.1.1. Sulfatase family, sequence homology and subcellular localization

Sulfatases are found in procaryotes and eucaryotes. Most of them share a substantial similarity at the protein level which extends over their entire length but is highest in the N-terminal third of their sequence (Franco *et al.*, 1995; Parenti *et al.*, 1997). This extensive sequence homology is shared by sulfatases from procaryotic, lower and higher eucaryotic cells. Thus, there are strong indications that the sulfatase genes belong to an evolutionarily conserved gene family (Fig.1.1). In the human genome, 17 sulfatases have been identified, eleven of which were characterised and found to show distinct sulfatase activities and/or subcellular localisation. A subgroup of seven human sulfatases were termed arylsulfatases due to their ability to cleave synthetic chromogenic or fluorogenic arylsulfates that are applied in enzyme assays and histochemistry (arylsulfatases A,B,C) or due to their striking similarity to arylsulfatase C (arylsulfatases D,E,F,G). At least eight human sulfatases are localized in the lysosomes, where they catalyse the degradation of glycosaminoglycans and sulfolipids. The arylsulfatase C (steroid sulfatase) is an integral membrane protein of the endoplasmic reticulum and the plasma membrane and is involved in cholesterol and steroid hormones metabolism. Arylsulfatases D, E and F are non-lysosomal membrane proteins that have been localized to the endoplasmic reticulum (arylsulfatases D and F) and Golgi apparatus (arylsulfatase E) (Parenti *et al.*, 1997). The physiological roles of arylsulfatases D, E and F are still to be determined, although there are indications that arylsulfatase E is possibly involved in vitamin K metabolism (Daniele *et al.*, 1998). The newly identified member of the sulfatase family Sulf1 (KiAA 1077) is localised on the cell surface and was shown to be

involved in regulation of Wnt signalling during embryo patterning (Dhoot *et al.*, 2001). By heparan sulfate desulfation this sulfatase triggers Wnt release from the proteoglycan, a step required for embryonic muscle specification.

Human sulfatases (from genome):														**Length (residues)**	**Signal peptide**	**FGly**
Arylsulfatase A	C	T	P	S	R	A	A	L	L	T	G	R	(Pos.69-80)	507	+	+
Arylsulfatase B	C	T	P	S	R	S	Q	L	L	T	G	R	(Pos.91-102)	533	+	+
Arylsulfatase C	C	T	P	S	R	A	A	F	M	T	G	R	(Pos.83-94)	583	+	n.d.*
Arylsulfatase D	C	T	P	S	R	A	A	F	L	T	G	R	(Pos.89-100)	593	+	n.d.
Arylsulfatase E	C	T	P	S	R	A	A	F	L	T	G	R	(Pos.86-97)	589	+	n.d.
Arylsulfatase F	C	S	P	S	R	S	A	F	L	T	G	R	(Pos.79-90)	591	+	n.d.
Arylsulfatase G	C	T	P	S	R	A	A	F	L	T	G	R	(Pos.181-192)	688	?	n.d.
N-Acetylgalactosamine 6-sulfatase	C	S	P	S	R	A	A	L	L	T	G	R	(Pos.79-90)	522	+	n.d.
Nacetylglycosamine 6-sulfatase	C	C	P	S	R	A	S	I	L	T	G	K	(Pos.91-102)	552	+	n.d.
Iduronate sulfatase	C	A	P	S	R	V	S	F	L	T	G	R	(Pos.84-95)	550	+	n.d.
Sulfamidase	C	S	P	S	R	A	S	L	L	T	G	L	(Pos.70-81)	502	+	n.d.
Sulf 6	C	S	P	S	R	A	S	L	L	T	G	R	(Pos.84-95)	525	+	n.d.
KIAA 1077	C	C	P	S	R	S	S	M	L	T	G	K	(Pos.87-98)	871	+	n.d.
KIAA 1247	C	C	P	S	R	S	S	I	L	T	G	K	(Pos.88-99)	870	+	n.d.
Sulf 3	C	C	P	S	R	A	A	M	W	S	G	L	(Pos.70-81)	526	+	n.d.
Sulf 4	C	T	P	S	R	S	Q	F	I	T	G	K	(Pos.96-107)	573	+	n.d.
Sulf 5	C	T	P	S	R	S	Q	L	L	T	G	R	(Pos.93-104)	569	+	n.d.
Lower eukaryotic sulfatases:																
Coturnix coturnix(Qsulf1)	C	C	P	S	R	S	S	M	L	T	G	K	(Pos.87-98)	869	+	n.d.
Hemicentrotus pulcherrimus	C	T	P	S	R	S	A	I	M	T	G	R	(Pos.100-111)	551	+	n.d.
Strongylocentrotus purpuratis	C	T	P	S	R	S	A	I	V	T	G	R	(Pos.115-126)	567	+	n.d.
Heliocidaris erythrogramma	C	T	P	S	R	S	A	I	M	T	G	R	(Pos.106-117)	559	+	n.d.
Volvox carteri	C	C	P	S	R	T	N	L	W	R	G	Q	(Pos.72-86)	649	+	+
Chlamydomonas reinhardtii	C	C	P	S	R	T	N	L	W	R	G	Q	(Pos.73-84)	646	+	n.d.
Neurospora crassa	C	C	P	A	R	V	S	L	W	T	G	K	(Pos.89-100)	639	+	n.d.

* n.d.- not determined

Figure 1.1. Sequence similarities among members of the sulfatase protein family downstream of the cysteine to be converted to FGly.
Amino acid residues, conserved in more than 60% of human sulfatases are indicated by blocks. Sulfatases V-Z and arylsulfatase G are putative sulfatases predicted from the human genome. KIAA 1077 is the human ortholog of Qsulf1 from *Coturnix coturnix*.

1.1.2. Single sulfatase deficiencies, their cause and consequences

The biological importance of human sulfatases is highlighted by the manifestation of eight known inherited metabolic diseases that are caused by the specific deficiencies of single sulfatases. Each of these disorders is associated with impaired desulfation of specific substrate metabolites and with their lysosomal storage (Table 1). For example, Arylsulafatase A is involved in catabolism of cerebroside 3-sulfate, a major constituent of myelin sheaths, and some other related sulfolipids that share a galactose 3-sulfate residue (Kolodny and Fluharty, 1995). Deficiency of this sulfatase is the cause of metachromatic leukodystrophy, a lysosomal storage disorder that leads to severe neurological symptoms because of extensive demyelination in the central and peripheral nervous system. Arylsulfatase B is involved in degradative pathways of dermatan sulfate and chondroitin 4-sulfate (Neufeld and Muenzer, 1995). These glycosaminoglycans are constituents of proteoglycans existing in the extracellular matrix. Deficiency of arylsulfatase B leads to mucopolysaccaridosis VI, a lysosomal storage disorder with a variety of clinical phenotypes such as dwarfism, skeletal abnormalities, cloudy corneas and heart failure (Neufeld and Muenzer, 1995). Deficiency of arylsulfatase C leads to X-linked ichthyosis, a relatively mild disease of the skin with increased levels of cholesterol sulfate in the stratum corneum (Ballabio and Shapiro, 1995). These distinct single sulfatase deficiencies emphasise the high substrate specificity of the sulfatases and their limited functional redundancy. The resulting clinical phenotypes are ranging from extremely severe and life threatening with early death to reduced quality of life and reduced life expectancy.

The deficiency of sulfatases in case of each syndrome is caused by different genetic defects. For example, one of the most common mutations associated with a mild form of metachromatic leukodystrophy is substitution of proline 426 by leucine in arylsulfatase A (ASA). The resulting deficiency of ASA is caused by its instability in the lysosomes. Recently it was shown that instability of the mutated enzyme is caused by its defective oligomerisation in the lysosomes which drastically increases the vulnerability of the enzyme to degradation by lysosomal proteases (von Bülow *et al.*, 2002). Point mutations of the corresponding sulfatase genes are also the cause of many other sulfatase deficiencies, among them various mucopolysaccaridoses.

Table 1. Human sulfatases, their subcellular localisation, natural substrates and metabolic disorders caused by their deficiencies[a]

Sulfatase	Subcellular localization	Natural substrate	Disease
Arylsulfatase A	Lysosomes	Cerebroside 3-sulfate	Metachromatic leukodystrophy
Arylsulfatase B	Lysosomes	Dermatan sulfate, chondroitin 4-sulfate	MPS VI (Maroteaux-Lamy)
N-acetylgalactosamine 6-sulfatase (Galactose 6-sulfatase)	Lysosomes	Keratan sulfate, Chondroitin 6-sulfate	MPS IV A (Morquio A)
N-Acetylglucosamine 6-sulfatase	Lysosomes	Heparan sulfate, Keratan sulfate	MPS III D (Sanfilippo D)
Iduronate sulfatase	Lysosomes	Dermatan sul fate, Heparan sulfate	MPS II (Hunter)
Sulfamidase (Heparan *N*-Sulfatase)	Lysosomes	Heparan sulfate	MPS III A (Sanfilippo A)
Glucuronate 2-Sulfatase[b]	Lysosomes	Heparan sulfate	Not established
N-Acetylglucosamine 3-sulfatase[b]	Lysosomes	Heparan sulfate	Not established
Arylsulfatase C (Steroid sulfatase)	Endolasmic reticulum, plasma membrane	3β-Hydroxysteroid sulfates	X-linked ichthyosis
Arylsulfatase D	Endoplasmic reticulum	Unknown	Not established
Arylsulfatase E	Golgi apparatus	Unknown	Chondrodysplasia punctata
Arylsulfatase F	Endoplasmic reticulum	Unknown	Not established
Sulf1 (KIAA 1077)	Extracellular	Heparan sulfate	Not established

[a] The eponyms of the various mucopolysaccharidoses (MPS) are given in parentheses.
[b] Sulfatase not yet identified (only characterised biochemically)

1.2. Multiple sulfatase deficiency and Cα-formylglycine

In addition to single sulfatase deficiencies, a rare autosomal recessive disorder called multiple sulfatase deficiency (MSD) is known, in which the activities of all known sulfatases are severely decreased. First cases of MSD were described by Austin (Austin, 1963; 1965) in two siblings. Since then quite a number of MSD patients were described (Kolodny and Fluharty,

1995). The clinical phenotype of MSD combines the features of single sufatase deficiencies, like metachromatic leucodystropy, mucopolysaccaridosis and ichthyosis. Common features are rapid neurologic deterioration and developmental delay. MSD is a relatively rare disease, with an estimated prevalence, for example, in Australia of 1 in 1,4 million births, compared to 1 in 92,000 and 1 in 235,000 for metachromatic leukodystropy and mucopolysaccaridosis type IV, respectively (Meikle et al., 1999).

Single sulfatase deficiency syndroms are caused by mutations in the corresponding sulfatase genes. In case of each syndrome the activity of only one sulfatase is decreased, all the other sulfatases are active within the normal range. On the contrary, the sulfatase deficiencies in case of MSD must have one common reason for all sulfatases. Studies in cultured cells from MSD patients have shown that synthesis of sulfatase polypeptides is normal but their catalytic activity is severely decreased, and for some of the sulfatase polypeptides their stability as well (Steckel *et al.*, 1985; Eto *et al.*, 1987). Synthesis of inactive sulfatases was observed, even when expressing wild type ASA, ASB or ASC-cDNAs in MSD cells (Rommerskirch and von Figura,1992). Therefore, it was concluded that sulfatases require posttranslational activation, that is missing in case of MSD. And, indeed, it was shown first for ASA and ASB that at the position 69 and 91 of the two polypeptides, respectively, where the cDNAs predict a cysteine residue, a novel amino acid, 2-amino-3-oxopropanoic acid (Cα-formylglycine, FGly, see Fig.2) was present (Schmidt *et al.*, 1995). ASA and ASB synthesized in MSD cells retained the cysteine predicted by the cDNAs sequences. FGly was found at a position where the cDNA sequences of all known eukaryotic sulfatases predict a cysteine within a highly conserved sequence. This sequence of 12 amino acids is shown in Fig.1. Later the presence of FGly was shown also for lower eukaryotic (Selmer *et al.*, 1996) and prokaryotic (Miech *et al.*, 1998; Dierks *et al.*, 1998b) sulfatases. This demonstrates that the FGly residue is conserved in prokaryotic and eukaryotic members of the sulfatase family and can be generated by oxidation of either a cysteine (eucaryotes and prokaryotes) or, as will be explained below, a serine residue (prokaryotes only).

A hypothetical mechanism of oxidation of the cysteine to FGly is shown in Fig.1.2. This mechanism starts with an oxidation step (formation of a thioadehyde) that is followed by a hydrolysis step (formation of FGly).

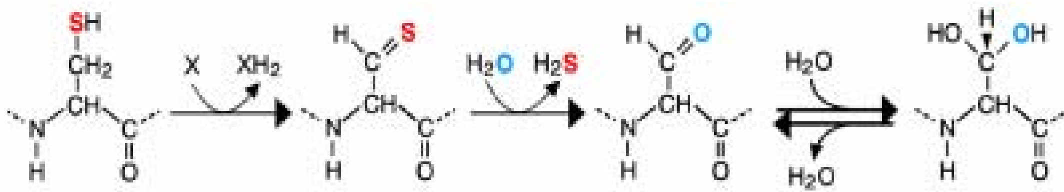


Figure 1.2. Hypothetical scheme of oxidation of cysteine into FGly-hydrate (von Figura et al., 1998).
In the first, enzymatically catalyzed, step the thiomethyl side chain of the cysteine is oxidized to thioaldehyde with participation of an unknown hydrogen-acceptor X. The two following reactions, hydrolysis of the thioaldehyde with release of hydrogen sulfide and the formation of the aldehyde hydrate, may occur spontaneously. The aldehyde hydrate can be detected in the native protein, where it is stabilized by a calcium cation and other functional groups (Fig.3).

The sulfatases synthesized in MSD cells are not absolutely inactive. In all MSD patients examined, low residual sulfatase activities could be detected that are attributed to a residual activity of the FGly generating enzyme. This most likely means that complete absence of FGly formation, resulting in synthesis of totally inactive sulfatases, is embryonically lethal (Recksieck *et al.*, 1998; Knaust *et al.*, 1998), which agrees with the important functions of Sulf1 (KiAA 1077) and most likely also Sulf2 (Fig.1) during embryonic development (see above).

1.2.1. Role of formylglycine in sulfate ester hydrolysis

The crystal structures of human sulfatases ASA and ASB were determined at 2.1 Å and 2.5 Å, respectively. The FGly was shown to be located in the active site cavity (Fig.1.3) and represents part of a metal binding site with an octahedrally coordinated metal ion (Lukatela *et al.*, 1998; Bond *et al.*, 1997). The 3-D structure of the bacterial sulfatase from *Pseudomonas aeruginosa* (PAS), resolved at 1.3 Å, established the metal ion in the active site as a Ca^{2+} (Boltes *et al.*, 2001). The structures of pro- and eukaryotic active sites of sulfatases are identical within the error limits and show topographic similarity to that of alkaline phosphatase. The side chain of FGly is superimposable to that of the catalytically essential serine 102 in the alkaline phosphatase active center (Kim, Wyckoff, 1998).
Structural and enzymatic studies of ASA mutants, in which the FGly was replaced by a serine or alanine (Recksiek *et al.*, 1998; von Bülow *et al.*, 2001) and the recent solution of the X-ray structure of PAS (Boltes *et al.*, 2001) provided insight into the actual mechanism of sulfate ester hydrolysis. The electron density map of the FGly side chain showed the presence of two

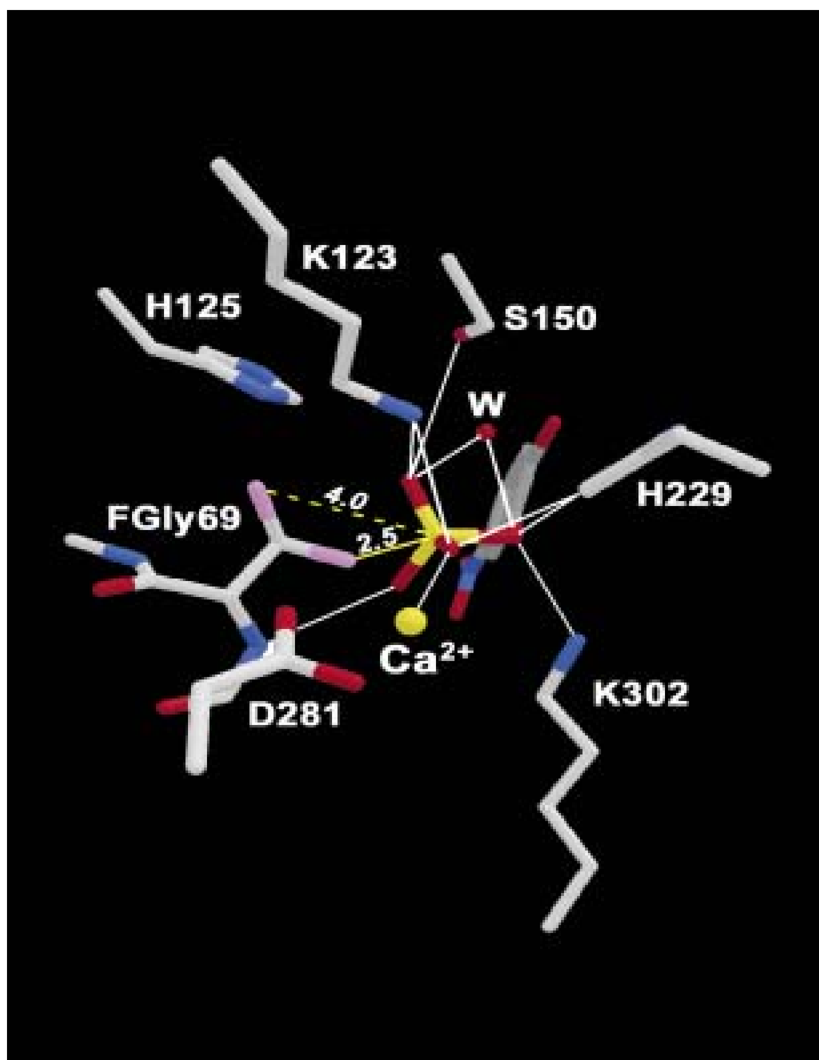


Figure 1.3. **Active centre of human arylsulfatase A** (von Bülow et al., 2001; Boltes et al., 2002). The sulfate group of the substrate is positioned in the active center and coordinated by lysine 123, serin 150, histidine 229, lysine 302, a water molecule (W) and a Ca^{2+} ion. The nucleophilic attack on the sulfur atom by one of the hydroxyl groups of FGly69-hydrate (distance 2.5Å) is facilitated by deprotonation of this hydroxyl group by aspartate 281.

hydroxyl groups bonded to Cβ in FGly 51, the key catalytic site chain in PAS, corresponding to FGly 69 in ASA and FGly 91 in ASB. Thus, the FGly residue in the active enzyme is present as a FGly-hydrate (Fig.1.4*A*). One of the two hydroxyl groups of the geminal diol is oriented towards the core of the protein, while the other one is positioned close enough towards the sulfate sulfur atom to start a nucleophilic attack on the substrate (Fig.1.3 and Fig.1.4*B*). The nucleophilicity of this hydroxyl group is enhanced by its coordination to a calcium cation and by the possibility of a proton transfer to an aspartate carboxyl group. The elecrophilicity of the sulfur in the substrate is enchanced by interaction of the sulfate oxygens with positively charged side chains of histidines and lysines and with the calcium cation. An

S_N2 substitution reaction with a pentacoordinated sulfur intermediate and heterolytic cleavage of the S-O ester bond results in a covalently sulfated substrate intermediate (Fig.4*C*). Elimination of the sulfate takes place with participation of the second hydroxyl group of the geminal diol and is required for regeneration of FGly. Finally the aldehyde hydrate is regenerated by an incoming water molecule (Fig. 1.4*D*).

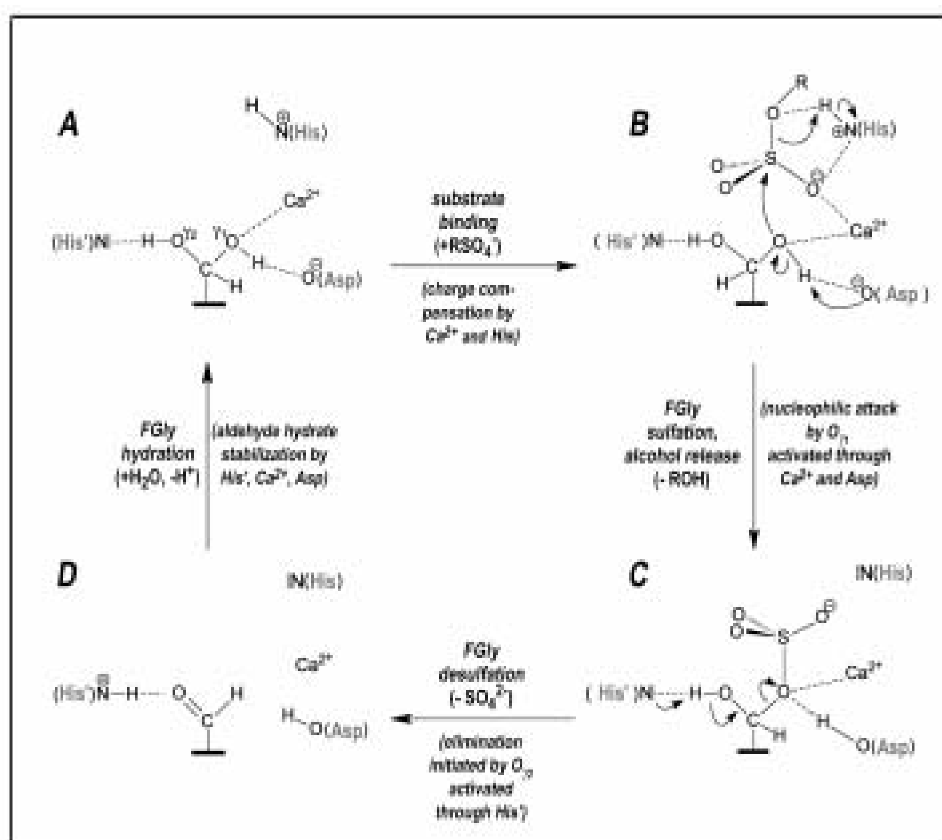


Figure 1.4. Scheme of the proposed catalytic mechanism for sulfate ester hydrolysis (Boltes *et al*, 2001).
In the geminal diol of FGly-hydrate, one of the alcohol groups, $O^{\gamma 2}$, is oriented towards the core of the protein and is stabilized by the neighbouring His, Arg and Lys residues (A). The other alcohol group $O^{\gamma 1}$ is very close (2.5Å) from the sulfate sulfur atom to start a nucleophilic attack on the sulfur and its nucleophilicity is enhanced both by its coordination to the calcium atom and the possibility of a proton transfer to the carboxyl group of the aspartate, which would be stabilized in turn by the divalent cation. (B). The orientation of the sulfate with the side of the sulfate tetrahedron facing FGly69-$O^{\gamma 1}$ group is stabilized by the charged side chains of histidine and lysine residues.
After substrate desulfation the acohol is released and diffuses out of the catalytic pocket. The sulfate is now covalently bound to the enzyme (C) and additionally coordinated to the calcium atom through two of its oxygen atoms. The C-O bond in the FGly-sulfate ester is polarized through all the contacts of the sulfate group to positively charged residues, favoring its elimination.
The final step of the reaction is elimination of sulfate and regeneration of the aldehyde (D). The aldehyde hydrate is regenerated by addition of a water molecule and is stabilized by hydrogen bonds to histidine, arginine nd the calcium atom.

The mechanism, as described, is based on the structural analyses of sulfatases and their substrate complexes (Bond et al., 1997; Lukatela et al., 1998; von Bülow et al., 2001; Boltes et al., 2001) and was also confirmed by kinetic studies of numerous active site mutants (Recksiek et al., 1998; Waldow et al., 1999; Knaust et al., 1998). The ASA-C69S and the corresponding ASB-C91S mutants allowed to trap the sulfated FGly intemediate (Fig.1.4*C*). The sulfate could no longer be eliminated from this intermediate, since the second hydroxyl group was absent in these mutants (Recksiek et al., 1998).
The stuctural similarity of sulfatases and in particular of their active site regions, strongly suggests that the proposed catalytic mechanism is common for all sulfatases. The key function of the FGly residue in this mechanism explains the critical role of the posttranslational generation of this residue in the biogenesis of enzymatically active sulfatases.

1.2.2. Formylglycine modification motif

In experiments with *de novo* synthesized ASA fragments it was shown that a sequence of 16 amino acid residues,encompassing positions –4 to +11 with respect to the cysteine to be modified (Fig.5), is essential and sufficient for the formation of FGly (Dierks *et al.*, 1997, 1999). Formation of FGly was observed with the same efficiency as that of the control after transferring these 16 residues to a heterologous protein (Dierks *et al.*, 1999). Analysis of different deletion- and substitution- mutants of ASA could show that a sequence motif consisting of 12 consecutive residues starting with the cysteine to be modified is nesessary to reach optimal FGly formation (Dierks *et al.*, 1999). This sequence consists of an essential core motif **C**xPxR and an auxiliary motif xxxL/MTGR/K/L. Apart from the cysteine, the key residues of the core motif are the proline and the arginine in position +2 and +4, respectively, which can be found in all known and putative members of the sulfatase family. The auxiliary motif (position +5 to +11 after cysteine to be modified) is playing a supporting but not an essential role in FGly formation. Even complete substitution of the highly conserved amino acid residues LTGR (Fig.1.5) by an AAAA-tetrapeptide led only to 50% reduction of FGly formation (Dierks *et al.*, 1999). It is proposed that this sequence of seven amino acid residues facilitates presentation of the core motif to the modifying enzyme.

1.2.3. Formation of formylglycine by luminal components of the endoplasmic reticulum

The cysteine residue, encoded in the sulfatase genes, is incorporated into the nascent sulfatase polypeptide chain during translation (Dierks *et al.*, 1997). The cysteine is converted to FGly

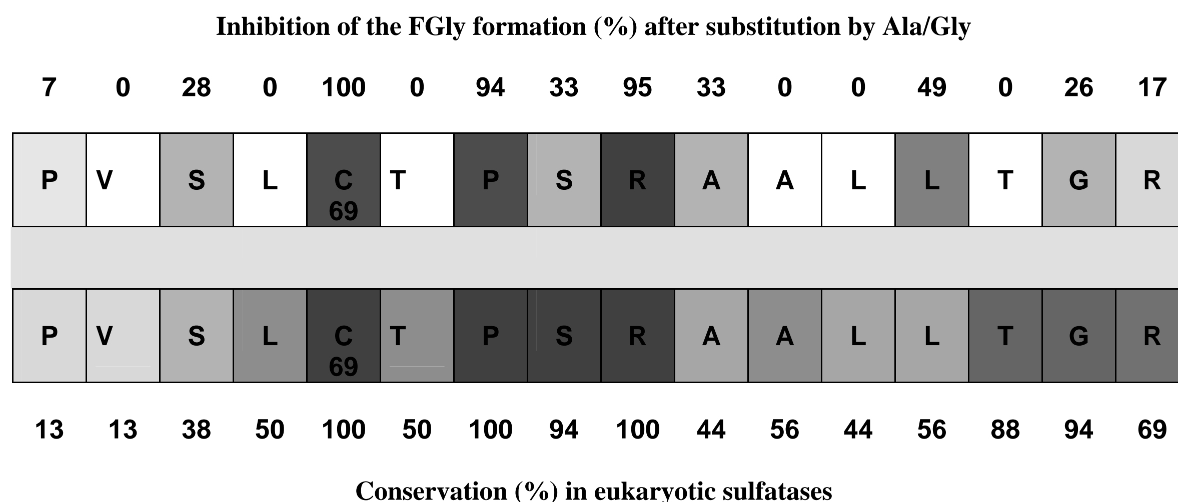


Figure 1.5. A short linear sequence determines the FGly formation (Dierks *et al*., 1999).
The 16-mer sequence of the human Arylsulafatase A is essential and sufficient for the modification of Cys69. Inhibition of FGly formation after substitution of each single amino acid residue by alanine or glycine is given in the upper row in numbers and indicated by different intensity of grey tones. In the lower row, intensity of grey tones and per cent values indicate the conservation level of the amino acid residues among eukaryotic sulfatases. The comparison of the two rows demonstrate that all three residues, C69, P71, R73, which are essential for FGly formation are 100% conserved among all eukaryotic sulfatases.

during or shortly after protein translocation into the endoplasmic reticulum (ER), as could be shown in an *in vitro* translation/translocation system comprising import competent dog pancreas microsomes (Dierks *et al*., 1997; 1998a). It was shown that, in an arrested translocation intermediate, the cysteine 69 of ASA was accessible inside the microsomes. However, FGly formation was observed only after releasing the nascent chain from the ribosome by puromycin (Dierks *et al*., 1997). Thus, FGly formation occurs after or at a late stage of cotranslational protein translocation.

In a recent study of *in vitro* FGly formation (Fey *et al*., 2001) it was investigated whether the components of the FGly-generating machinery are part of the ER membrane or the ER lumen or both. The dog pancreas microsomes were treated with increasing detergent concentrations and separated into supernatant and pellet fractions by centrifugation. Increasing concentrations of the detergent solubilized increasing amounts of FGly-generating activity that was recovered in the supernatant fraction. Under conditions that selectively extract the luminal components but leave membrane components in the pellet fraction, almost 100% of FGly generating activity was recovered in the supernatant and virtualy no activity remained in the pellet fraction. Thus, the FGly modifying machinery is part of the soluble components of the ER lumen referred to as reticuloplasm.

In the same study it was shown that *in vitro* formation of FGly by reticuloplasm does not depend on the presence of the signal peptide sequence in the sulfatase substrate. Furthermore, the FGly-generating enzyme was characterised with respect to some basic biochemical properties such as pH optimum, which was unexpectedly high (pH 10-10.5). Kinetically controlled conditions were established for assaying FGly formation *in vitro*. The FGly forming activity showed typical enzymatic properties with a characteristic temperature dependence (activation energy 61 kJ/mol) and sensitivity to inhibitory peptides (Fey *et al.*, 2001; see also Results, paragraph 3.3). Reticuloplasm as a source for the FGly-generating enzyme and the *in vitro* assay for detection of its activity are the basis for the functional and chromatographic characterisation of the so far unknown modifying protein on the way to its eventual identification.

1.3. FGly generating machinery in prokaryotes

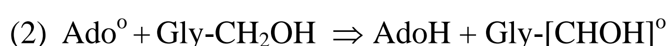
Mammalian sulfatases are involved in the turnover of endogenous sulfated substrates (von Figura et al., 1998). On the other hand, sulfatases of lower eukaryotes, like algae or fungi, and of bacteria, are expressed under conditions of sulfur starvation and function in sulfate scavenging from exogenous substrates (Dodgson et al., 1982). Due to conservation of the FGly modification motif, most of the sulfatases encoded in various eubacterial genomes are predicted also to undergo FGly modification by oxidation of cysteine. This was shown experimentally for the arylsulfatase from *Pseudomonas aeruginosa*, a member of the cysteine-type sulfatases. Even after strong overexpression in *E.coli* this cytosolic sulfatase was quantitatively converted to the active FGly-bearing enzyme (Dierks *et al.*, 1998b). Thus, the *E.coli* cytosol must contain the FGly modifying machinery. This machinery is expressed even under excessive inorganic sulfate supply conditions and thus is independent of the sulfur status of the cell, in contrast to expression of the sulfatase structural genes, as shown for *P.aeruginosa* and *K.pneumoniae* (Beil *et al.*, 1995; Murooka *et al.*, 1990; Dodgson *et al.*, 1982).

The other well characterised bacterial sulfatase, the arysulfatase of *K.pneumoniae*, is a serine-type sulfatase that carries an FGly residue generated by oxidation of a serine rather than a cysteine (Miech *et al.*, 1998). In contrast to the cytosolic cysteine-type sulfatases, serine-type sulfatases are located in the periplasm (Murooka *et al.*, 1990; Schirmer and Kolter, 1998). The key FGly motif (SXPXR) and also the auxiliary downstream element (LTG) are conserved in serine-type sulfatases (Dierks *et al.*, 1999).

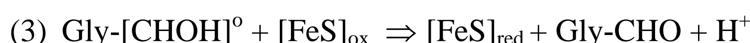
Despite these similarities, bacteria have two different pathways for FGly generation from cysteine and serine, respectively. This is indicated by two observations: first, substitution of the cysteine to be modified in *Pseudomonas* sulfatase by serine totally blocks FGly formation (Dierks *et al*., 1998b); second, expression of an active, FGly containing *Klebsiella* sulfatase in E.coli essentially requires coexpression of the *Klebsiella atsB* gene (Szameit *et al*., 1999), while the genomic background of *E.coli* is sufficient for expressing an active and modified *Pseudomonas* sulfatase. It was shown recently, that AtsB, which is a iron-sulfur protein, is located in the cytosol and is interacting directly with the serine-type FGly modification motif, which allows to consider the possibility that AtsB itself is the oxidising enzyme converting serine to FGly (Marquordt *et al*., 2002).
Some iron sulfur proteins related to AtsB were shown to have enzymatic redox functions by generating radical species by reductive cleavage of S-adenosylmethionine (SAM) through an unusual FeS center that is also present in AtsB (Sofia et al., 2001; Cheek and Broderick, 2001). A possible reaction sequence for AtsB-mediated FGly formation is outlined in Scheme 1 . Transfer of an electron from the reduced FeS center to S-adenosylmethionine leads to its reductive cleavage (step 1). The generated deoxyadenosyl radical abstracts a hydrogen atom from the substrate, i.e. the Ser72 side chain, under formation of deoxyadenosine and a substrate radical (step 2). The single electron of this radical is then accepted by the FeS center, leading to its re-reduction, under formation of FGly (step 3).

Scheme 1: Proposed mechanism for AtsB-mediated FGly-formation (Marquordt et al., 2002).

(1) $[FeS]_{red} + AdoMet^{+} \Rightarrow [FeS]_{ox} + Methionine + Ado^{o}$



(2) $Ado^{o} + Gly\text{-}CH_2OH \Rightarrow AdoH + Gly\text{-}[CHOH]^{o}$



(3) $Gly\text{-}[CHOH]^{o} + [FeS]_{ox} \Rightarrow [FeS]_{red} + Gly\text{-}CHO + H^{+}$

$AdoMet^{+}$ = S-adenosylmethionine

Ado^{o} = 5’-deoxyadenosyl radical

AdoH = 5’-deoxyadenosine

$Gly\text{-}CH_2OH$ = Serine

Gly-CHO = FGly

As FGly formation from a serine is most likely a single enzymatic reaction, AtsB may suffice for FGly formation in serine-type sulfatases. On the contrary, the cysteine-modifying enzyme could not be identified to date in bacteria. A transposon-mutagenesis approach failed (in

E.coli), indicating that the cysteine-modifying system is either redundant or essential, i.e. required for other purposes apart from FGly-modification of sulfatases.

1.4. Goals and experimental approach

Like in bacteria, the cysteine-modifying enzymatic machinery awaits its identification also in eukaryotes. Genetic approach in lower eukaryotes failed so far, since expression of ASA as a human reporter sulfatase in *Schizosaccharomyces pombe* or *Aspergillus nidulans* led to misfolded aggregates without activity and, most likely, without FGly (Voigt *et al.*, unpublished). Whereas these two organisms express endogenous sulfatases, *S. cerevisiae* does not carry sulfatase genes in its genome. The only so far identified component of a bacterial FGly generating system, the iron-sulphur protein AtsB, is required for FGly formation specifically in serine-type sulfatases. In eukaryotes serine-type sulfatases are missing. AtsB has only very weak homologs encoded in mammalian genomes, the best being viperin (Chin and Cresswell, 2001), an interferon-inducible antiviral protein that locates to the endoplasmic reticulum, where eukaryotic FGly formation (from cysteine) occurs. Viperin, however, shows only 26% identity with the N-terminal third of AtsB and only one of AtsB's three predicted iron-sulphur centres is conserved. Viperin could be excluded to be required for FGly-formation in eukaryotes (see Discussion).

Thus, the development of an approach for characterisation and identification of the components of FGly generating machinery in eukaryotes is necessary and of high priority. In this work the biochemical approach towards characterisation and identification of the FGly generating enzyme is undertaken. The deficient FGly formation in all human sulfatases in case of multiple sulfatase deficiency syndrom indicates that the FGly generating mechanism is common for all human sulfatases and the disease is caused by a genetic defect of the component(s) involved. Identification of this/these component(s) will help to understand the nature of this severe and life-threatening disease and pave the way for a possible therapy.

Identification of sequence determinants directing conversion of cysteine to FGly (Dierks et al., 1999) allowed to develop substrates suitable for assaying FGly generating activity *in vitro.* The recent localisation of the eukaryotic FGly generation machinery to the lumen of the endoplasmic reticulum (Fey et al., 2001) led to the preparation of these luminal content (reticuloplasm), which is a good enzyme source, allowing its chromatographic purification in the absence of the membrane components and detergent. Development of a fluid-phase *in vitro* FGly modification assay that is independent of a substrate synthesis (*in vitro* translation)

and its translocation across membranes should allow determination of FGly generating activity under kinetically controlled conditions. All these findings provide a solid base for the undertaken biochemical approach.

The main goals of this study were:

- to use non-canine microsomes as a source for FGly generating enzyme;
- to optimise and standardize the *in vitro* FGly modification assay;
- to find out whether the FGly generating system consists of one or more components;
- to investigate the influence of different cofactors on the FGly generating activity *in vitro*;
- to establish the chromatographic protocols (such as ion exchange, gel filtration, hydrophobic interaction, dye affinity chromatography, *etc*.) for separation of endoplasmic reticulum luminal proteins;
- to investigate the behaviour of FGly generating activity on the single chromatographic columns and to optimise the chromatographic conditions (binding and elution) for each purification protocol to ensure the best recovery of FGly generating activity;
- to establish the affinity purification protocol using the ASA 16-mer peptide, comprising the FGly generating motif (Fig.5) as a ligand; and to optimise the binding and elution conditions for the affinity purification;
- to establish the optimal FGly generating activity purification strategy using combination of single purification protocols in order to get a highly pure protein fraction containing FGly generating activity;
- to identify the candidate proteins from the purified FGly generating activity fraction using MALDI-TOF mass spectrometric analysis.

2. MATERIALS AND METHODS

2.1. Appliances

Analytic balances type 1602 MP and 1265 MP	Sartorius, Goettingen
Analytical and preparative HPLC:	
SMART-system with following columns:	Amersham Biosciences, Freiburg
Gel filtration columns:	
Fast Desalting PC 3.2/10 (3.2 x 100 mm)	Amersham Biosciences, Freiburg
Superdex® 200 PC 3.2/30 (2.4ml)	Amersham Biosciences, Freiburg
Anion exchange columns:	
MonoQ® PC 1.6/5 (0.1ml)	Amersham Biosciences, Freiburg
MonoQ® HR 5/5 (1ml)	Amersham Biosciences, Freiburg
MonoQ® HR 10/10 (8ml)	Amersham Biosciences, Freiburg
Cation exchange column:	
MonoS® PC 1.6/5 (0.1ml)	Amersham Biosciences, Freiburg
Hydrophobic interaction column:	
Phenyl Superose® PC 1.6/5 (0.1ml)	Amersham Biosciences, Freiburg
Reversed Phase columns:	
Aquapore RP-300 (C8, 2.1 x 220 mm)	
µPeak C2/C18 PC 3.2/3 (C2/C18, 2.1 x 30 mm)	Amersham Biosciences, Freiburg
UV-detectors for SMART-system:	
µPeak Detector	Amersham Biosciences, Freiburg
Vision Workstation for Perfusion chromatography	PerSeptive Biosystems, USA
UV-Detector for Vision Workstation:	
FLUOR-305	PerSeptive Biosystems, USA
Intelligent Dark Box II, Las-1000+	Fuji, Japan
Ice machine	Ziegra, Isernhagen
Centrifuges:	
Eppendorf centrifuge Type 5415C and 5402	Eppendorf, Hamburg
Table ultracentrifuge TL-100	Beckmann, München
Ulracentrifuge L8-70M	Beckmann, München

Cold Centrifuge J-21C and J2-MC	Beckmann, München
Labofuge GL	Heraeus Sepatech
Rotors:	
JA 20	Beckmann, München
Ti 45, Ti 60, Ti 70	Beckmann, München
TLA 45,	Beckmann, München
TLA-100.3	Beckmann, München
Electrophoresis chambers for agarose gels and polyacrilamide gels	Workshop of the Institute
Liquid scintillation counter 1900TR	Packard, Frankfurt/Main
Gel dryer	Bio-Rad, Hilden
Magnetic mixer	IKA, Works, INC., Wilmington/USA
MALDI-TOF Mass Spectrometer, REFLEX III	Bruker Daltonics, Bremen
Microwave oven	Siemens, München
pH-Meter	Beckmann, München
Photometer, UV 160 A	Shimadzu, Kioto/Japan
UV-hand lamp (365/254nm), Type 5415 and 5402	Eppendorf, Hamburg
Vacuum concentrator model 100H	Bachhofer, Reutlingen
Vortex-Genie	Scientific Industries, inc. Bohemia/USA

2.2. Chemicals

Acetonitril, HPLC grad	Backer
Acetic acid	Roth
30% Acrylamide	Roth
Agar	Sigmar
Ammonium chloride	Merck
Ammonium acetate	Fluka
Ammonium sulfate	Merck
α-cyano.4.Hydroxyl cinnamic acid	Bruker Daltonik
2% Bisacrylamide	Roth

Bromphenolblue	BioRad
Calcuim chloride	Merck
Coomassie colloidal blue	Roth
Dimethylsulfoxid, ultrapure (DMSO)	Merck
Deoxy Big Chap	Calbiochem
Dithiothreitol, ultrapure	Serva
Dinithrophenylhydrazine (DNPH)	Fluka
Ethylendiamintetraacetic acid-disodium salt (EDTA)	Merck
Ethanol	Roth
Ethanolamine	Sigma
Ficoll	Sigma
Formaldehyde 37%	Merck
Glycerine	Merck
Glycine	Roth
Guanidine hydrocloride	Fluka
H_2O HPLC grade	Baker
HEPES (N-2-Hydroxyethylpiperasin-N'-2-ethansulfonic acid)	Merck
Hydrochloric acid	Roth
Jodoacetic acid	Sigma
Magnesium sulfate	Merck
Magnesium chloride	Merck
2-ß-mercaptoethanol	Sigma
Methanol	Merck
Potassium acetate	Merck
Potassium chloride	Merck
Potassium hydrogenphosphate	Merck
Potassium hydroxide	Merck
Potassium thiocyanate	Merck
Puromycine dihydrochloride	Sigma
Scintilation liquid	Roth
Silver nitrate	Sigma

Sodium acetate	Merck
Sodium azide	Sigma
Sodium carbonate	Merck
Sodium chloride	Roth
Sodium dodecyl sulfate (SDS)	Sigma
Sodium dihydrogen carbonate	Merck
Sodium hydrogen phosphate	Merck
Sodium hydroxide	Merck
Sodium phosphate	Merck
Sucrose	Merck
TEMED N,N,N`,N`-tetramethylendiamin	Sigma
Threefluoroacetic acid	Sigma
Tris-(hydroxymethyl)-aminomethan (Tris)	Merck

2.3. Radioactively labelled substances

L-(35S)-Methionine, water solution 1000 Ci/mmol	Amersham-Buchler, Braunschweig

2.4. Enzymes, Standards and Antibiotics

Klenow-polimerase	New England Biolabs, Bad Schwalbach
Restriction endonucleases	New England Biolabs, Bad Schwalbach
λ/*Hind*III-Standard	Gibco BRL, Eggenstein
1-kb-DNA-Ladder	Gibco BRL, Eggenstein
Ampicilline	Serva, Heidelberg
Precision Protein Standards for PAGE electrophorese	BioRad
Trypsin modified, sequencing grade	Roche

Oligonucleotides	NAPS, Göttingen

2.5. Protein determination, Translation and Transcription Kits, DNA Kits

BioRad Protein Assay	BioRad, München
QIAprep Plasmid Miniprep Kit	Qiagen, Hilden
QIAquick PCR-purification	Qiagen, Hilden
ABI PRISM Big-Dye Terminator Cycle Sequencing Ready Reaction	Applied Biosystems
RIBO-MAX Large Scale RNA Production System – SP6	Promega
Retuculocyte Lysate L416A Nuclease free	Promega

2.6. Protease Inhibitors

Protease inhibitors coctail for use with mammalian cell and tissue extract	Sigma
PEFA-Bloc	Roth

2.7. Chromatographic media and affinity matrixes

Affi-gel 10 Gel	BioRad
Dye affinity matrixes:	
Cibacron Blue agarose	Sigma
Reactive Blue-4 agarose	Sigma
Reactive Brown-10 agarose	Sigma
Reactive Green-19 agarose	Sigma
Reactive Red-120 agarose	Sigma
Reactive Yellow-86 agarose	Sigma
Concanavalin A Sepharose	Amersham Biosciences, Freiburg
Heparin immobilized on crosslinked 4% beaded agarose	Sigma

Chromatographic media for Vision workstation:	
POROS® HQ, QE, PI ion exchange media	PerSeptive Biosystems, USA
POROS® 20 HP, PE, ET hydrophobic interactions media	PerSeptive Biosystems, USA

2.8. Other materials

Cellophan folie	Pütz-Folien, Taunusstein-Wehen
Centrifuge tubes:	
JA-20 Polypropylene	SCI Science Service, München
Ti-45 Polypropylene	Beckmann, München
Ti-60 Polypropylene	Beckmann, München
TLA 100.3 Polypropylene	Beckmann, München
Centrifuge adaptors for TLA 100.3	Beckmann, München
Filters with 35µm pore size	MobiTec, Göttingen
Hamilton syringes, 50-1000µl	Hamilton, Bonaduz/Switzerland
Laboratory glass vials	Schütt, Göttingen
One way pipette tips	Braun, Melsungen
Parafilm	American National Can, Neenah/USA
Pasteur pipettes	Schütt, Göttingen
Plastic tubes small (15ml) and large (50ml)	Greiner, Nürtingen
Polycarbonate centrifuge tubes for ultracentrifuge rotor TLA 100.3	Beckmann, München
Reaction tubes: 0,5 ml	Sarstedt, Braunschweig
1,5 and 2 ml	Greiner, Nürtingen
Plastic columns (2,5ml; 6,5ml and 12,5ml)	MobiTec, Göttingen
Scalpel, steril	Braun, Melsungen
Scintillation liquid Lumasafe Plus	LUMAC, the Netherlands

Liquid scintillation vials Zinsser, Germany

Ultrathimbles, UH 100/25 Schleicher & Schuell, Dassel

2.9. Antibodies

2.9.1 Primary antibodies

Name	Antigen	Immunised species	Purification	Reference
α PDI	PDI	Mouse, polyclonal	Serum	
α Sec61α	Sec61α	Rabbit, polyclonal	Serum	Prof. Dr. T.Rapoport, Boston/USA
α Calreticulin	Calreticulin	Rabbit, polyclonal	Serum	Dr.Phuc Nguyen Van, Goettingen
α Viperin	Viperin	Rabbit, polyclonal	Serum	Prof. Dr. P.Cresswell, USA

2.9.2. Secondary antibodies

Goat anti rabbit, HRP conjugated Dianova, Hamburg

Goat anti mouse, HRP conjugated Dianova, Hamburg

2.10. Bacterial cell culture media

Lurta-Bertami (LB)-Medium:
10g/l Bacto Trypton
5g/l Bacto Yeast Extract
5g/l NaCl
2.5g/l $MgSO_4$ x 7 H_2O (pH adjusted to 7.5 with NaOH)

Selection medium: +100 µg/ml Ampicilline

2.11. Bacteria cells

Stamm:	Source:
E.coli DH5α	Gibco BRL, eggenstein

2.12. Stock solutions and frequently used buffers

Ammoniumperoxodisulfat (APS) solution	20% (v/w)	Ammoniumpersulfat
7M ammonium acetate solution	7M	Ammonium acetate
50 x TAE solution	2M	Tris-HCl
	0.1M	EDTA (pH adusted to 8.0 with acetic acid)
PBS-buffer	137mM	NaCl
	3mM	KCl
	8mM	Na_2HPO_4
Pefa Block	0.5M	Pefablock in H_20
2 x Laemmli loading buffer	0.1M	Tris-HCl, pH 6.8
	2% (w/v)	SDS
	2% (v/v)	ß-mercaptoethanol
	20% (v/v)	Glycerol
	0.002% (w/v)	Bromphenolblue
10 x DNA loading buffer	30% (w/v)	Ficoll
	0.25% (w/v)	Brophenolblau
	0.25% (w/v)	Xylen Cyanol FF
	0.5M	EDTA, pH 8.0
		Phenol filled with TE buffer, pH 8.0
Phenol	0.1% (w/v)	Hydroxychinoin
Phenol/Chlorophorm/Isoamylalcohol: (25:24:1)		Prepared according to Sambrook *et al.*, 1989

2.13. Computers and software

Hardware:	Apple Power PC 7600/132	Apple Macintosh

	Apple laser writer 16/600 PS IBM compatible PC 486 Scan Jet 4c/T	 Hewlett Packard
Software:	AIDA Image Analyser v.3.10 Adobe® Photoshop® 5.5 Adobe® Ilustrator Windows 95 Windows XP Microsoft Office 95-2000 MASCOT Search Engine Canvas MC Draw Pro Aldus FreeHand 3.6 Cricket Graph WinCam 2.2 Image reader	RayTest Adobe Adobe Microsoft Microsoft Microsoft Mascot Canvas Apple Macintosh Apple Macintosh Apple Macintosh Cyberteck, Berlin Fuji Film

2.14. Analytical methods

2.14.1. SDS-Polyacrilamide Gel Electrophoresis (Laemmli et al., 1970)

The elecrophoretic separation of the proteins was performed through „high-Tris" discontinual SDS polyacrilamide gel electrophoresis. A system with vertical oriented glass plates (16 x 16 cm; 1 mm spacer) was used. The gels were prepared as follows: first the separating gel (12,5 – 17,5% acrylamide) was put between glass plates and covered with a layer of butanol. After polymerisation of the gel was completed, butanol was removed and the space between glass plates was dried with Watmann paper. Then the concentrating gel was put on top of the polymerised separating gel and the sample combs were introduced immediately. After approximately 15 min polymerisation was completed and the sample combs were removed. The glass plates with the gel between them were fixed inside the electrophoresis chamber and covered with electrophoresis buffer. Protein samples were mixed with Laemmli loading buffer 1:1, denatured by 950C and centrifuged with 14000 rpm. The supernatant was

introduced into the concentrating gel pockets. Electrophoresis was performed for 2-3 hours by the constant current of 50mA.

Electrophoresis buffer: SDS (w/v) 10g/10 l
Glycine (w/v) 144,27g/10 l
Tris (w/v) 60,53g/10 l

Ammoniumperoxiddisulfate (APS): 20% (w/v) in ddH2O

Stacking gel	
Ingredients	
30% Acrylamid solution: (ml)	0.4
1% bisacrylamid solution (ml)	0.275
ddH2O (ml)	1.175
0,5 M Tris-HCl, pH 6.8 (ml)	0.625
(10%) SDS (µl)	25
TEMED (µl)	2.5
(20%) APS (µl)	10

Separating gel	
Ingredients	15%
30% Acrylamid solution: (ml)	8.75
1% bisacrylamid solution (ml)	2.74
ddH2O (ml)	1.4
1,5 M Tris-HCl, pH 8.8 (ml)	4.3
(10%) SDS (µl)	175
TEMED (µl)	14.58
(20%) APS (µl)	58.33

2.14.2. Detection of proteins in polyacrylamide gels

2.14.2.1 Staining with Roti-Blue Colloidal Coomassie

Colloidal Coolmassie staining is one of the most sensitive staining protocols. Due to its colloidal properties the dye binds with high specificity to proteins and only minimal to the gel matrix. This allows visualisation of proteins separated by SDS-PAGE with sensitivity as high as 30ng of protein. Roti-Blue collodial Coomassie was purchased from Roth and staining was performed according to the protocol as follows. Immediately after completion of

electrophoresis gels were incubated in the fixing solution for 60 min, shaking. The staining solution was applied on to the gels for 60 min. In special cases the gels were stained for several days, which improved the sensitivity of the staining. After completion of staining gels were incubated in the washing solution for 5-10 min and then kept in the stabilising solution or prepared for drying in the drying solution.

Fixing solution: 20% (v/v) methanol
8,5% (v/v) o-phosphoric acid

Staining solution: 20% (v/v) methanol
20% (v/v) 5x concentrate Roti-Blue colloidal Coomassie

Washing solution: 25% (v/v) methanol

Stabilising solution: 20% (w/v) ammonium sulphate

Drying solution: 10% (v/v) glycerol
20% (v/v) ethanol

2.14.2.2. Detection of radioactively labelled polypeptides

For detection of radioactively labelled polypeptides polyacriamide gels were fixed in destaining solution for 15 min and then washed with H_2O for 30 min. Then the gels were dried between cellofan layers in the gel-dryer for 1,5 – 2 hours. For visualisation of the incorporated radioactive label the gels were exposed on to the „Imaging plate“ (Fuji) overnight by RT. The image on the plate was analysed by the program „Image reader“ (Fuji-Film, Vers. 1.4 E) with the help of the BAS 1000 phosphoimager (Ray-test).

2.14.2.3. Staining with silver (Schevchenko *et al*., 1996)

Staining of proteins separated on SDS-gel with silver allows quick and effective visualization of even small amounts of protein. After completion of electrophoresis gels were incubated with shaking in fixing solution for 1 hour. After that gels were washed 2 times for 20 min in 30% ethanol and equilibrated for 20 min in water. Equilibrated gels were washed for 1 min in 0.03% $Na_2S_2O_3$, washed with water and incubated in staining solution for 20 min at 4^oC. Then

the gels were washed in water twice for 30 seconds and developed in the developing solution until desired intensity of the bands was obtained. Developing was stopped by transferring the gels into a new chamber with 5% acetic acid. Gels were kept in 1% acetic acid or washed with water to prepare them for drying.

Fixing solution:	10% (v/v) acetic acid
	40% (v/v) ethanol
Staining solution:	0.2% (w/v) silver nitrat
	0,008% (v/v) formic aldehyde
Developing solution:	3% (w/v) sodium bicarbonate
	0,018% (v/v) formic aldehyde

2.14.2.4. Staining with Ruby Red fluorescent dye

Ruby red fluorescent dye was purchased from Bio-Rad and staining was performed according to the following protocol. After completion of electrophoresis the gels were washed for 30 min in 10% (v/v) methanol, 7% (v/v) acetic acid prior to staining. After removal of the washing solution gels were covered with Ruby Red fluorescent dye and incubated with gentle agitation for 3 hours or overnight. Then the gels were washed with 10% methanol, 7% acetic acid to decrease background fluorescence. Developing of the gels was performed on the Fuji-imager in the fluorescence mode.

2.14.3. Protein determination by „Bradford“ (Bradford, 1976)

Measuring of the protein concentration by Bradford method is based on the ability of Coomassie-staining to change its absorption maximum after binding to proteins from 465 nm to 595 nm. Quantitative measurement was performed with staining solution and according to the protocol „BioRad Protein Assay“ (Richmond, USA) spectrometrically by 595 nm.

2.14.4. FGly modification assay (Fey et al., 2001)

In the standard assay 7,5-30 eq reticuloplasm (purified chromatographic fractions, affinity columns flow through and eluates) was diluted to 87,5µl with OG buffer (no detergent) and incubated with 5µl of salt-washed RNCs and 7,5µl of 20mM puromycin (final concentration

in the assay 1,5mM) for 20-40min at 37°C. Incubation was stopped by adding 167 µl of preheated to 80°C RCM buffer and heating for 10 min at 80°C.
Variation to the standard protocol with respect to the source and amount of the modifying enzyme, assay volume, buffer conditions, incubation conditions and RNCs are indicated in the description of each experiment in the Result chapter.
Aliquots of assay samples (2,5µl) were routinely checked for recovery of the substrate polypeptide by SDS-PAGE and phosphoimaging (BAS 1000, Ray-test).

RCM buffer: 6 M guanidine hydrochloride
400 mM Tris-HCl, pH 8,6
10 mM EDTA

2.14.5. Peptide analysis for presence of FGly

After completion of the FGly modification assay the resulting mixture of modified and unmodified substrate ASA polypeptides was subjected to several further steps (see below) of chromatographic analysis in order to distinguish between these two forms of polypeptide and to quantify the amount of modified peptide as the percentage of total, *i.e.* modified plus unmodified peptide.

2.14.5.1. Reductive carboxymethylation (Schmidt *et al.* 1995)

To improve the efficiency of the following tryptic digestion of arylsulfatase A the disulfate bonds formed by cysteine residues of the polypeptide were reduced with DTT and denatured polypeptide was stabilized through carboxymethylation of free thiol groups by iodoacetic acid. To control the efficiency of following tryptic digestion BSA as a carrier protein with known digestion pattern was added to the samples where reaction was stopped with RCM buffer to reach the final concentration of 100 µg per ml. DTT was added to the mixture (final concentration 50 mM) and after removal of the oxygen by argon the mixture was incubated at 52oC for 60 min in the dark. After cooling the sample to RT iodoacetic acid was added (final concentration 150 mM) and the sample (oxygen was removed by argon) was incubated by RT for 30 min protected from light. The reaction was stopped by addition of 1M DTT to the final concentration of 150 mM. The samples were subjected to chromatographic desalting.

2.14.5.2. Desalting of carboxymethylated samples and tryptic digestion

In order to prepare the carboxymethilated samples for tryptic digestion, to remove salt and change the buffer, the gel filtration procedure was applied. Sephadex G-25 column (Fast Desalting PC 3.2/100) was equilibrated with trypsin reaction buffer on the SMART HPLC-system (Pharmacia). The sample was centrifuged 10 min by 14000 rpm to remove insoluble parts and the supernatant was applied on the column. Gel filtration was performed by flow rate of 100 ml per min with automatic fractionation and detection of absorption by 280-, 295- and 340nm wavelengths. The protein peak reached its maximum approximately at 2.5 min after injection and after completion of the run was automatically integrated by 280 nm wavelength over reference wavelength of 340 nm for quantification of the eluted protein.
For proteolytic fragmentation of the carboxymethilated and desalted proteins trypsin was added to final concentration 3% (w/w) and sample was incubated 16 hours at 37oC.

Trypsin reaction buffer:	10% (v/v)	Acetonitril
	50 mM	Ammonium acetate, pH 8.6

2.14.5.3. Peptide separation through „reversed phase“ chromatography (RP-HPLC)

Reversed phase chromatography allows effective and fast separation of proteins and peptides according to their hydrophobic properties. In order to distinguish between cystein and folmylglycine forms of tryptic peptides of ASA the peptides were separated on C8 column (Aquapore RP 300, Pharmacia) on the SMART system (Pharmacia). The column was installed on the SMART system with long column adaptor and equilibrated prior the run in buffer A (0,1% trifluoroacetic acid). The samples after completion of tryptic digestion were centrifuged at 14 000 rpm at RT to remove insoluble parts. The supernatant was applied on the column and the peptides were eluted with increasing concentration of buffer B (90% acetonitril, 0,1% trifluoroacetic acid) at the flow rate of 300 µl per min. Automatic fractionation was performed to collect bound as well as unbound to the column material.

2.14.5.4. Detection of radioactively labelled tryptic peptides by liquid scintillation counting

In order to detect radioactively labelled tryptic peptides 30 µl of each fraction was placed into scintillation counter vial and mixed with 1ml of scintillation liquid (LUMAC). Vials were shaked to allow thorough mixing of the radioactive samples with scintillation liquid and placed in to the liquid scintillation counter (1900TR, Packard). Counting was performed for 3

min by RT for each vial. The results were presented in cpm (counts per minute) units. To exclude chemoluminescence interfering effects the samples were counted the second time in 24 hours.

2.14.5.5. Quantitative determination of presence of aldehyde group by creation of peptidyl-2,4-dinitrophenylhydrazone (DNPH-assay) (Dierks et al, 1997)

Dinitrophenilhydrazine is a reagent which reacts specifically with aldehydes with formation of hydrazone complexes. Since folmyglycine is carrying an aldehyde group it would allow the separation of formyglycine form of tryptic ASA peptide from the cystein form since due to hydrasone derivatisation the hydrophobic properties of folrmylglycine form of the peptide change and this leads to the change of its retention time on RP-HPLC column. This fact was used for quantification of the formylglycine formation.

DNPH was dissolved to the end concentration of 50 mg/ml in 50% acetonitril, 0,5% TFA. The identified through liquid scintillation counting RP-HPLC purified peptide was dried in vacuum and dissolved in 25 µl of 0,5% TFA. 25µl of DNPH solution was added to the peptide and the mixture was incubated for 30 min at 37°C in the dark to allow hydrasone formation. Then the mixture was centrifuged for 2 min at 14000 rpm and applied on to RP-HPLC C18 column (µpeak C2/C18 PC 3.2/3, Pharmacia) on the SMART system. The column was equilibrated with buffer A (0,1% TFA) prior to sample application. The supernatant of the centrifuged sample was applied on the column and bound peptides were eluted with increasing concentration of buffer B (90% acetonitril, 0,1% TFA). 150µl of each of automatically collected fractions were counted in liquid scintillation counter .

2.14.6. Identification of proteins by Matrix Assisted Laser Distortion Ionisation/Time-of-Flight (MALDI-TOF) Mass Spectrometry

2.14.6.1. In gel digestion of proteins separated by SDS-PAGE

The stained bands were excised from the gel with a scalpel and transferred into a PCR cup. The excised bands could have been kept at 4°C for several days or frozen at –20°C.

The analysed bands were cut into approximately 1x1mm pieces and the staining was washed away by the following procedure:

- adding of 100µl of HPLC-grade water;

shaking the tube at 37°C for 30 min;
discarding supernatant;

- adding of 100µl of 25mM of NH_3CO_2 (neutralising solution);
  shaking the tube at 37°C for 30 min;
  discarding supernatant;

- adding of 100µl of 50% acetonitril, 25 mM NH_3CO_2;
  shaking the tube at 37°C for 30 min;
  discarding supernatant;

- adding of 100µl of 100% acetonitril;
  shaking the tube at 37°C for 30 min;
  discarding supernatant;

The washed gel pieces were dried at RT for 5 min. For in gel digestion 25µl ice cold trypsin solution were added. Trypsin was purchased specially from Promega: V5111, Seq. Grade Modified. Gel pieces covered with trypsin solution were kept on ice for 15 min. If in some samples the gel pieces were not completely covered with liquid, some µl of 25 mM NH_3CO_2 were added to cover them. For digestion the samples were incubated overnight (approximately 18 hours) in 37°C chamber.

2.14.6.2. Extraction of tryptic peptides from the gel pieces

During overnight incubation with trypsin the proteins in the gel pieces were cleaved by trypsin with the formation of tryptic peptides. For further analysis the peptides should have been extracted from the gel matrix. For that in the cup (first) and in the bottom of the incubation cup the holes were made with a needle. The cup was placed in the new intact Eppendorf tube and the liquid was forced into a new cup by centrifuging at 2500 rpm for 2 min in an Eppendorf centrifuge. The gel pieces were covered with 100µl of 1% TFA and shaked for 30 min at 37°C, then centrifuged at 2500 rpm for 2 min. Again the gel pieces were covered with 100µl of 50% acetonitril/1% TFA, shaked at 37°C and centrifuged at 2500 rpm for 3 min. The supernatant, containing tryptic peptides was dried in a vacuum concentrator (Speed Vac).

To resolve dried peptides 10µl of 0.1% TFA was added to the tubes, the tubes were shaked and to ensure recovery of the peptides the samples were subjected to ultrasound sonification. Tryptic peptide solution could be kept at –20°C.

2.14.6.3. ZipTip purification of the tryptic peptides before analysis by MALDI-TOF

Purification of the tryptic peptides with ZipTip is used to remove salts and other contaminants which could disturb the following mass spectromectical analysis. Before purification the ZipTip was equilibrated with 0.1% TFA/70% acetonitril by pipetting the solution in and out of the tip several times with following washing with 0.1% TFA.

Binding of the peptides to ZipTip matrix was performed by pipetting the solution through the tip for 5 times. The unbound components were washed away with 0.1% TFA. The peptides were eluted with 0.1%TFA/70% acetonitril solution. The eluate was used for MALDI-TOF mass spectrometrical analysis.

2.14.6.4. Sample/matrix preparation for MALDI-MS (drying droplet method)

For preparation of the matrix 1000µl of 50% acetonitril/0.1% TFA was added to 5 mg of α-cyano-4-hydroxycinnamic acid with following shaking for 1 min at RT and ultrasound sonification for 5 min. The solution was centrifuged in an Eppendorf centrifuge at 13000 rpm for 5 min. The supernatant was used for cocristallization with the sample. For best results this saturated matrix solution had to be made freshly every day.

For cocristallisation of the sample and matrix 1µl of the matrix solution was carefully mixed with 1µl of the sample with the pipette. 1µl of the mixture was applied carefully onto MALDI target (without touching the target with the tip) and dried in the air at the room temperature.

2.14.6.5. Obtaining the mass spectra on MALDI-TOF

MALDI positive ion mass spectra were obtained with a Bruker Daltonik Reflex III, using 337nm nitrogen laser, with 200ns extraction delay. Spectra were obtained as averages of 100 laser shots.

2.15. Preparative methods

2.15.1. *In vitro* expression

2.15.1.1. In vitro transcription with SP6-RNA polymerase (Fey et al., 2001)

Run off transcripts lacking a stop codon were obtained by in vitro trascription of pTD31/AlwNI or pMB2/AlwNI with SP6 polymerase using the Ribomax system (Promega). Prior to transcription the linearized template ends were blunted with Klenow DNA polymerase for 15 min at 22°C.

Mix for blunting DNA ends with Klenow DNA Polymerase

DNA (1µg/µl)	12 µl
Klenow DNA polymerase	0,39 µl
5x Buffer	6,25µl
H_2O	3,13 µl

Afterwards 6,25µl of rNTPs mix and 3,13µl SP6 enzyme were added to the sample and it was incubated for 3 hours at 37^{0}C.

2.15.1.2. In vitro translation in reticulocite lysate

„Run-off" transcripts, obtained by *in vitro* transcription can be used as mRNA in different translation systems (Krieg and Melton, 1984). In this work the endogenous mRNA and tRNA depleted reticulocyte lysate from Promega company was used. With help of this *in vitro* protein synthesis system it was possible to test whether relevant cystein residue in mammalian arylsulfatase A was modified into Ca-Formylglycin (FGly). Radioactively labelled proteins were synthesized *in vitro* in presence of ^{35}[S]-Methionine. Synthesized proteins were analysed by separating by SDS-gel electrophoresis to control the efficiency of translation and used for in vitro FGly generating assay . For performing of *in vitro* translation mRNA-transcripts were introduced into premix (s. table 2) and incubated for 15 min at 26°C. After translation the mix was diluted with 850µl SB-buffer and ribosome-nascent chain complexes (RNCs) were isolated according to protocol by Neuhof (Neuhof et al., 1998) by density gradient centrifugation on 1 ml sucrose cushion in 13x51-mm polycarbonate tubes (Beckman, table ultracentrifuge, rotor TLA 100.3, 100 000 rpm, 60 min, 2°C). The supernatant was immediately removed and ribosomal pellet was resuspended in 20µl OG-buffer. RNCs pellet was then shock-frozen in liquid nitrogen and could be kept at –80oC.

Translation premix: 12 µl ^{35}[S]-Methionine
1,5 µl run-off transcript (MB2/*Alw*NI)
3 ml Amino acids mix without methionine

75 µl reticulocyte lysate
58,5µl nuclease free H_2O

OG-Buffer: 125 mM sucrose
50 mM Hepes pH 8.0
150 mM potassium acetate
1 mM magnesium acetate
2,5 mM DTT

SB-Buffer: 25 mM Hepes-KOH, pH 8.0
2,5 mM magnesium acetate
5% glycerol
200 mM potassium acetate
1 mM DTT

Cushion: 50mM Hepes-KOH, pH 7,5
5 mM magnesium acetate
500 mM potassium acetate
500 mM sucrose

2.15.2. Preparation of rough microsomes from cow pancreas/testis

Cow pancreas/testis was obtained from the local slaughter house directly after the cows were slaughtered to ensure the freshness of material. Immediately after separation the pancreas was washed several times in wash buffer (10 mM Tris, pH 7,5; 150 mM sodium chloride). Pancreas was transported and kept on ice in puffer A (Pi mix 1:5000).
All the following steps were performed at 4°C.
One preparation was made from 50g of pancreas.
Pancreas was cleaned from fats, blood and connective tissue and thoroughly chopped with razor blades. Chopped pancreas was mixed with 3 volumes of buffer A (Pi mix 1:500). The mix was homogenized with the help of boring machine in a Teflone potter. The homogenized material was pelleted by centrifugation in Falcon 50 ml tubes by 500g for 10 min. The supernatant was filtered through the gaze, transferred into a new tube and subjected to the

centrifugation by 3000g for 10 min. The resulting supernatant was centrifuged again in JA 20 tubes in JA20 rotor for 10 min by 20000g. The supernatant was used for the following centrifugation step.

The final centrifugation step was performed in Ti 45 or Ti 60 rotors (Beckman). The polycarbonate tubes for corresponding rotors were half filled with the supernatant and the cushion buffer was carefully undelayered to the bottom of the tube. Prepared tubes were centrifuged in the corresponding rotors for 3,5 hours at 45000 rpm (Ti 45 rotor) or 3 hours at 50 000 rpm (Ti 60 rotor) at 4^oC.

After completion of centrifugation the supernatant and cushion were carefully removed and pellet was resuspended in the membrane buffer. For determination of protein concentration the aliquot was taken. Resuspended pellet (= rough endoplasmic reticulum microsomes suspension) was aliquoted, shock-frozen in liquid nitrogen and kept at 4^oC.

Protein concentration in the microsomal membranes is expressed in equivalents. 1 equivalent = 0.02 OD_{280nm}.

Buffer A:	50 mM Hepes pH 7,6
	50 mM potassium acetate
	6 mM magnesium acetate
	1 mM EDTA
	250 mM sucrose
	0,5 mM PEFA-Block
	1 mM DTT
	1:500 or 1:5000 Protease inhibitor cocktail
Cushion:	50 mM Hepes pH 7,6
	50 mM potassium acetate
	6 mM magnesium acetate
	1 mM EDTA
	1,3 M sucrose
	1 mM DTT
Membrane buffer	50 mM Hepes pH 8,0
	250 mM sucrose

2,5 mM DTT

2.15.3. Detergent extraction of luminal components of the ER from rough pancreas microsomes (Fey *et al*., 2001)

2.15.3.1. Extraction on the analytical scale

Luminal components of the endoplasmic reticulum can be obtained by detergent extraction of the rough microsomes. To wash away the membrane buffer (see 3.2.1) microsomal suspension was diluted 1:2 with OG buffer and microsomes were pelleted by centrifugation for 7 min at 45000 rpm at 4°C in TLA 45 rotor in table ultracentrifuge. The pellet was then resuspended in OG buffer and DBC was added to reach final concentration of 2.5 mM. Extraction was performed by incubation on ice for 30 min. Membrane components were pelleted by centrifugation in TLA 100.3 (Beckman) rotor in table ultracentrifuge for 30 min at 68000 rpm at 4°C. Supernatant (reticuloplasm), representing the lumenal components of microsomes was taken out. Reticuloplasm was used directly for further analysis or aliqioted, shock frozen in liquid nitrogen and kept at –80°C.

2.15.3.2. Extraction on the preparative scale

For preparative extraction 12 ml reticulolasm were mixed with 13 ml OG buffer in a Ti60/Ti70 polypropylene tube and centrifuged in Ti 60 rotor (Beckmann) for 15 min at 45 000 rpm at 4°C. The supernatant was discarded and the pellet was resuspended in OG buffer to the final volume of 11.4 ml using glass homogeniser (1 ml or 7 ml). Then DBC was added to the sample to the final concentration of 2.5mM. Extraction was performed on ice for 45 min. Membrane components were pelleted by centrifugation in Ti60/Ti70 rotor for 80 min at 4°C at 50 000 rpm.

2.15.4. Liquid chromatography on SMART and VISION station systems

Different liquid chromatographic protocols allow separation of proteins according to their different properties, such as surface charge, hydrophobicity, size etc. In this work liquid chromatographic separation of reticuloplasm proteins was performed on the SMART system (Pharmacia) and VISION perfusion chromatography system (PerSeptive Biosystems).

SMART™ system is designed for optimal micropurification of biomolecules and provides high resolution and high recovery of separated molecules and their biological activity. The key to high recoveries and resolution is the fully integrated „miniaturized“ system. Small volume flow paths in combination with small dimension columns minimize dead volumes, unwanted dilution effects and non-specific adsorption. All these features make the system ideal for performing analytical chromatographic runs. In this work we used SMART system for establishing analytical purification protocols and also for some preparative applications (see below).

VISION Workstation for Perfusion Chromatography is designed for Perfusion Chromatography® flow-through particle chromatography – a patented new technology which performs bioseparations 10 to 100 times faster than conventional HPLC or LC systems without loss of capacity or resolution. Due to very high flow rates and high capacity of the system the runs could be performed in a very short time which made optimisation of the running conditions very convenient and allowed separation and purification of large amounts of peptides and proteins. In this work we used VISION station for preparative separation of reticulolasm proteins and also for purification of peptides.

2.15.4.1. Ion exchange chromatography

Ion exchange liquid chromatography allows effective separation of native proteins according to differences in their surface charge. Depending on the charge of the functional groups of the column matrix there are two main groups of ion exchange columns: anion exchangers and cation exchangers. In this work strong cation and anion exchangers were used.

2.15.4.1.1. Analytical separation of reticuloplasm proteins on the MonoQ PC® 1.6/5 strong anion exchange column on the SMART system

MonoQ PC® 1.6/5 column (Pharmacia) is a strong anion exchanger based on a beaded hydrophilic polymer. The charged group of the gel is $–CH_2$ $–N^+$-$(CH_3)_3$. The maximum protein/peptide capacity is in the range of 0.5-3 mg depending on the type of the sample and running conditions. The practical loading range is 0.5-500µg.

To prepare the sample for anion exchange chromatography it should be dissolved in the application buffer to reduce salt concentration, since high salt can prevent binding of proteins to the column matrix. Reticuloplasm was concentrated 3x fold in the Amicon concentrator and diluted with buffer A (20 mM Tris pH 8.0, 2,5 mM DTT) to the starting volume. The

MONOQ column was installed on to the SMART system and equilibrated with buffer A prior to the run. The sample was applied on the column at the flow rate of 100 µl per minute and bound proteins were eluted with increasing concentration of buffer B (20 mM Tris pH 8.0, 2M NaCl, 2,5 mM DTT). Practically all the proteins could be eluted within 20 min gradient from 0 to 37,5% of buffer B. Fractions of 100µl of flow through and of 50µl during the gradient were collected automatically.
Variations of the runs concerning buffer composition and sample load are described in the Result chapter.

2.15.4.1.2. Analytical separation of reticuloplasm proteins on the MonoS PC® 1.6/5 strong cation exchange column on SMART system

MonoS PC® 1.6/5 column (Pharmacia) is a strong cation exchanger based on a beaded hydrophilic polymer. The charged group of the gel is $–CH_2SO_3^-$. The maximum protein/peptide capacity is in the range of 0.5-3 mg depending on the type of the sample and running conditions. The practicar loading range is 0.5-500µg.
Preparation of the sample for cation exchanger run is similar to that of anion exchanger. Reticuloplasm was concentrated 3x fold in the Viva Spin concentrator and diluted with buffer A (20 mM MES pH 5.5, 2,5 mM DTT) to the starting volume. The MONOS PC 1,6/5 (0,1 ml) column was installed on to the SMART system and equilibrated with buffer A prior to the run. The sample was applied on the column at the flow rate of 100 µl per minute and bound proteins were eluted with increasing concentration of buffer B (20 mM MES pH 5,5, 2M NaCl, 2,5 mM DTT). Practically all the proteins could be eluted within 20 min gradient from 0 to 37,5% of buffer B. Fractions of 100µl were collected automatically.
Variations of the runs concerning buffer composition and sample load are described in the Result chapter.

2.15.4.1.3. Preparative separation of reticulolasm proteins on the MonoQ HR 5/5 and MonoQ HR 10/10 strong anion exchange columns on SMART system

MonoQ HR 5/5 (1 ml) and MonoQ HR 10/10 (8 ml) columns allow separation of proteins on the preparative scale. The charged group on the gel matrix is the same as that of the MonoQ PC 1.6/5 column. Protein capacity of the columns is in the range of 20-80 mg and 160 – 400 mg, accordingly . We could separate proteins from up to 5ml (40 ml) reticuloplasm in one run on these columns. To protect the columns from contaminations and to ensure their long life I

some cases the reticuloplasm was subjected to the detergent removal procedure prior to the run. For that retuculoplasm was mixed with detergent Bio-Beads® SM hydrophobic interaction adsorbent beads (Bio-Rad) in proportion of 5g of beads for every 25 ml reticuloplasm solution and the mixture was incubated shaking for 2 hours at 4°C. After that the beads with bound detergent were pelleted by centrifigation at 14000 rpm at RT and detergent-free reticulolasm was used for further analysis . Further separation of the sample is similar to that for the analytical anion exchange run (see 2.15.4.1.2). The columns were installed on the SMART system using long column adaptor. Prior to the run columns should have been equilibrated with 10 volumes buffer A (for buffers composition see 2.15.4.1.2), 10 volumes buffer B and 10 volumes buffer A consecutively. Sample was applied on the column through super loop 10 or 50 ml (Pharmacia). The run was performed at the flow rate of 2000 µl per minute. Bound proteins were eluted with gradient of buffer B from 0 to 37,5% during 40 min. Due to the very high flow rate and long time of the runs the buffer pumps of SMART system should have been filled with buffer several times during the run. Nevertheless this fact did not cause gradient disturbances, which could be controlled through the slope of conductivity curve (see Results chapter). Fractions of fixed volume of 1 ml for flow through and 0.5 ml during the gradient (1,5 ml for the first 30 min and of 1 ml during the gradient for MonoQ HR 10/10 column) were collected automatically.
Variations in sample volumes and buffer composition are described in the Result chapter.

2.15.4.2. Hydrophobic interactions chromatography (HIC)

Hydrophobic interactions chromatographic separation of proteins is based, like in reversed phase chromatography (RPC), on differences in proteins hydrophobicity. Since hydrophobic interaction matrixes are less hydrophobic then RPC-media, their require milder elution conditions. Therefore, the risk of irreversible protein denaturation is reduced, which allows separation of proteins in their native state with high recoveries of biological activity. Under favourable conditions for hydrophobic interactions, such as high salt concentrations, the exposed hydrophobic regions on a protein adsorb to the hydrophobic ligands on the matrix. Proteins are selectively eluted by a gradual reduction in salt concentration.

2.15.4.2.1 Analytical separation of reticulolasm proteins on Phenyl Superose column on SMART system

Phenyl Superose® Precision Column 1.6/5 (Pharmacia) is designed for fast high-resolving HIC. The hydrophobic ligand is a phenyl group, covalently linked to Superose 12 (12% highly cross inked agarose beads). The column has volume of 0.1 ml and can be used for separation of up to 3mg of protein depending on the type of sample and running conditions with practical loading range of 0.5-1500µg of protein.

To ensure binding of proteins to the column matrix the salt concentration in the sample should have been brought to that of buffer A (50 mM potassium phosphate, pH 7.0; 2M $(NH_4)_2SO_4$). For that 100-500µl of reticuloplasm were subjected to concentration and buffer exchange in Viva Spin concenrators (800µl). Phenyl Superose column was installed on the SMART system and equilibrated with 10 volumes of buffer B (50 mM potassium phosphate, pH 7.0) and 10 volumes of buffer A, consecutively. Sample was applied on the equilibrated with buffer A column and bound proteins were eluted in 20 min with increasing from 0 to 100% gradient of buffer B. The run was performed at flow rate of 50µl per minute with detection of absorption by 280-, 295- and 340nm wavelengths. Fractionation was performed automatically, flow trough and eluted proteins were collected in 100µl fractions.

Variations of the runs concerning buffer composition and sample load are described in the Result chapter.

2.15.4.2.2. Preparative separation of reticuloplasm proteins by HIC on the PE column on the VISION station

The buffer system, column equilibration and preparation of reticuloplasm for run on VISION station was the same as that for the SMART system.

The column (2.1mm x 100 mm) was packed with POROS-PE hydrophobic interactions media which consists of cross-linked poly(styrene-divinylbenzene) flow-through particles. The particles are surface coated with cross-linked polyhydroxylated polymer. This coating is further functionalised with phenyl ether groups. The column volume was 1.6 ml and protein capacity 5-10 mg/ml.

The run was performed at the flow rate of 2000µl/min and gradient of 0 to 100% of buffer B was achieved in 5 min. The run was completed in 10 min and the fractions of 500µl were collected automatically. Absorbance at 280 and 340nm wavelengths were detected.

Variations in sample load and buffer composition are described in Results chapter.

2.15.4.3. Gel filtration (size exclusion) chromatography on Superdex 200 column on the SMART system.

Gel filtration or size exclusion chromatography is based on separation of the biomolecules according to their size. In this work we performed analytical gel filtration runs on the Superdex 200 PC 3.2/30 column (Pharmacia) on the SMART system.

The column matrix is a composite of dextran and cross-linked agarose with particle size of approximately 13µm. The molecular weight range for the optimal separation of globular proteins is 10 000-600 000 and exclusion limit is a molecular weight of 1.3×10^6. Column volume is 2.4 ml and protein separation capacity is maximum 10mg but the practical loading range is up to 2.5 mg.

Prior the run the column was equilibrated with running buffer (150mM KAc, 125 mM sucrose, 1 mM $MgCl_2$, 50 mM Hepes pH 8.0, 2.5 mM DTT) for 20 min at flow rate of 100µl/min. To ensure high resolution the very small sample volume should have been used. For that sample was concentrated before the run in Viva Spin concentrator in Eppendorf table centrifuge at RT at 14000 rpm to 50 µl. The sample was applied on the column and the run was performed at constant flow of 40µl/min. Fractions of 100µl were collected automatically. Variations in sample composition are described in Results chapter.

2.15.5. Affinity chromatography

Affinity purification was performed on the Affi Gel 10 affinity matrix (Bio-Rad) with immobilized peptides.

2.15.5.1. Immobilization of peptides on the activated Affi Gel 10 affinity support

Affi Gel 10 is a complementary affinity support that offers rapid and high efficiency coupling for all ligands that have a primary amino group. Affi Gel 10 is a N-hydroxysuccinimide ester of a derivatized cross-linked agarose gel bead support and it couples to ligands spontaneously in aqueous and non-aqueous solution. Amide bonds couple the protein or peptide ligand to the terminal carboxyl group of the neutral 10-atom spacer arm of Affi Gel 10.

Coupling was performed according to the protocol supplied by Bio-Rad. All the steps of coupling procedure were performed at 4°C.

1-5 ml of the gel suspension was placed into 2.5-12.5 ml Mobicol plastic column. The liquid was sucked away with the syringe and the gel was washed with 3 vol cold isopropanol, 3 vol cold H_2O. The remaining liquid was removed by syringe. 5mg of peptide per 1ml of the gel

support were dissolved in 1ml of 200mM HEPES, pH 7.5. Peptide solution was applied on the washed gel and the coupling was performed with rotation for 24 hours.

After coupling was finished, the flow through with uncoupled peptide was collected and tested for presence of the peptide to estimate efficiency of coupling (see 2.15.5.2).

To block any active esters which have not been coupled the gel was incubated with rotation for 1 hour with 1 ml of 200 mM ethanolamine pH 8.0, 100mM HEPES pH 7.5 solution per 1 ml of the gel.

Finally the gel with immobilized peptide was washed with 10 vol PBS buffer. The gel was stored in PBS + 0.02% sodium azide at 4°C.

2.15.5.2. Test of coupling efficiency by RP-HPLC on SMART system

To test the efficiency of coupling of the peptide ligand to Affi Gel 10 affinity support the aliquots of 2µl were taken from the 5mg/ml peptide solution before coupling and the flow through of the affi-column after coupling. The aliquots were mixed with 16µl of HPLC buffer A and 2µl of HPLC buffer B. The samples were applied on the RP-HPLC C18 column (µpeak C2/C18 PC 3.2/3, Pharmacia) on SMART system. Absorbance at 214, 280 and 295 nm was detected. Bound peptide was eluted with 20 min gradient of buffer B from 0 to 100%. The peptide peak was collected by automatic peak fractionation and after the run the peak area was estimated by integration of 214 nm absorbance curve over background.

Peptide coupling efficiency was estimated by comparison of the peptide peak area before and after coupling.

2.15.5.3. Binding of proteins to the peptides immobilized on the affinity support

Total reticuloplasm and partly purified by liquid chromatography protocols proteins could be further purified on the affinity support with immobilized peptides.

The affinity gel suspension was placed on a Mobicol column and equilibrated with 5 vol of the incubation buffer (incubation buffer composition varied from experiment to experiment, for details see Results chapter). The protein containing sample was mixed with the equilibrated affinity gel and incubated for 2-2.5 hours at 4°C with rotation. After that the flow through was collected and tested for FGly generating activity.

2.15.5.4. Elution of FGly generating activity from the affinity matrix

Prior to elution the unbound proteins were washed away from the column with 10 vol of incubation buffer. Weakly unspecificly bound proteins could be eluted with 2M NaCl, 20 mM Tris, 2.5 mM DTT. Washing with this high salt buffer did not elute FGly generating activity. After high salt wash the column was washed again with 3 vol of incubation buffer. Then 2 column volumes of the elution buffer were applied and the column was incubated for 10 min – 3 hours, depending on the type of the elution, at 4°C with shaking or rotation. After that the eluate was collected and, depending on the type of elution buffer, either desalted or dialysed.

2.15.5.5. Desalting of the affinity eluate on the PD10 gel filtration column

When the high chaotropic salt elution conditions were chosen for elution of FGly generating activity from the affinity matrix, the eluate was desalted on the PD10 gel filtration column. PD10 gel filtration columns allow fast and effective desalting and buffer exchange in the sample without separation of proteins.

The column was equilibrated with 3 vol of OG buffer. 1.6ml of eluate was applied onto the column and washed down with 1.3ml of OG buffer. The proteins were eluted with 2.5 ml of OG buffer.

2.15.5.6. Concentration and dialysis of the affinity eluate in the ultrathimbles UH 100/25

The high salt and peptide eluates from the affinity column were concentrated and dialysed using ultrathimbles UN 100/25 (Schleicher&Schuell) with cut off of 25kDa. The sample was placed in the ultrathimble and concentrated with the help of the vacuum pump at 4°C. Dialysis was performed together with concentration by putting the ultrathimble in the 50 ml Falcon tube with dialysis buffer. To avoid large surface contact and the protein loss, the sample was put in the ultrathimble in the volume not more then 1 ml and refilled during the concentration.

When the sample was concentrated to 200-300µl the concentration was stopped and the sample was dialysed for 10-16 hours against the buffer (buffer composition varied depending on the type of the eluate) at 4°C in a glass vial with stirring.

Dialysed eluate was used for detecting of FGly generating activity and for separating the proteins on SDS-PAGE.

For details of sample nature, buffer composition etc., see the Results chapter.

2.15.6. Separation of proteins on Con A Sepharose

Con A Sepharose (Amersham Bioscience, Freiburg) is Concanavalin A coupled to Sepharose 4B by the cyanogen bromide method. Concanavalin A (Con A) is a lectin which binds to molecules which contain α-D-mannopyranosyl, α-D-glucopyranosyl and sterically related residues. Con A Sepharose is used for separation and purification of glycoproteins, polysaccarides and glycolipids.

Con A Sepharose purification was performed according to the protocol provided by Pharmacia Biotech.

The Con A Sepharose gel slurry was applied on to Mobicol plastic column. The gel was washed with 3 vol of buffer 1 and 3 vol of buffer 2 consecutively. To ensure effective binding the proteins should have been dissolved in buffer 2 prior to application on to the Con A matrix. After sample application the column was incubated with shaking for 30 min at 4^oC. The flow through with the unbound proteins was collected and the column was washed with 3 vol of buffer 2. Elution of bound proteins was performed by adding 2 vol of buffer 3 and incubation for 5 min at 37^oC shaking.

Buffer 1:
0.5 M KCl
50 mM HEPES pH 7.4
1 mM MgCl2
2.5 mM DTT

Buffer 2:
0.5 M KCl
50 mM HEPES pH 7.4
1 mM MgCl2
2.5 mM DTT
1 mM MnCl2
1 mM CaCl2

Buffer 3:
0.5 M KCl
50 mM HEPES pH 7.4
1 mM MgCl2
2.5 mM DTT
1 mM MnCl2
1 mM CaCl2

0.5 M Methyl Mannopyranoside

2.15.7. Separation of proteins on the Heparin agarose.

The gel suspension was placed on 2.5 ml Mobicol column and equilibrated with 5 vol of OG buffer without KAc. The protein sample should have been desalted before being applied on to the column to ensure binding to the heparin matrix. Buffer exchange in the sample was performed by concentration in the Viva Spin concentrators.

Binding of proteins to heparin agarose was performed at 4^oC for 30 min with rotation. After collecting of the unbound proteins in the flow through the gel was washed with 3 vol of incubation buffer (OG buffer without KAc). Bound proteins were eluted with 1M NaCl or 0,5M $(NH_4)_2SO_4$ in OG buffer. For elution the column was incubated with elution buffer for 30 min at RT with rotation.

2.15.8. Separation of proteins on the dye affinity matrices

Dye affinity matrixes consist of agarose beads with coupled synthetic dyes, which are mimicking cofactors and natural substrates of the enzymes. Prior to binding of proteins the columns were washed with 10 volumes of OG buffer without salt. Binding was performed in the MobiCol plastic columns at 4^oC, rotating, for 1 hour. After binding was complete, the unbound material was collected and column matrixes with bound proteins were washed with 3 volumes of the incubation buffer (OG buffer without salt). Elution was performed by incubating the columns for 15 min with OG buffer containing 1M NaCl.

2.16. Working with DNA

2.16.1.Cell culture

2.16.1.1. E.coli culture

E.coli cells were cultivated in LB liquid medium at 30^oC. To ensure constant and equal uptake of air the vials were shaked at 240 rpm. For selection and cultivation of transformed cells 100µg/µl of ampicilline were added to the medium.

To start new culture the glycerol-frozen cells were used.

2.16.1.2. Preparation of the glycerol cultures

Exponentially grown cultures from E.coli cells were mixed with 0.25 volume of 80% sterilized glycerol and shock-frozen in liquid nitrogen. The cultures were kept at –80°C.

2.16.2. Preparation, purification and digestion of DNA

2.16.2.1. Analytical isolation of plasmid DNA from E.coli cells (Quiagen handbook, April 1997)

Small amounts of plasmid DNA were isolated according to the protocol provided by producer of the buffer system used for preparation.

100 µg/ml of Ampicilline were added to 5 ml LB-medium and cells were incubated overnight by 37°C with shaking at 240 rpm. 2 ml of this bacterial culture was pelleted for 5 min by 14 rpm and sediment was resuspended in 250µl of ice-cold P1 buffer. After addition of 250µl of P2 buffer vials were incubated for 5 min by RT. 350ml buffer N3 were added, mixed and sample was precipitated on ice for 5 min. After sedimentation for 10 min by 14000 rpm at RT the clear supernatant was transferred on the mini-column and centrifuged for 1 min by 14000 rpm. The flow through was discarded and bond to the filter DNA was centrifuged for 1 min at 14000 rpm with 750µl of PE buffer. The supernatant was again discarded and sample was centrifuged for 1 min without addition of buffers to remove remaining ethanol. The elution of DNA was performed with 100µl distilled H_2O.

2.16.2.2. Preparative isolation of DNA from E.coli cells

Larger amounts of plasmid DNA were isolated according to the protocol of the producer of the buffer system used.

200 ml of the grown bacterial culture was centrifuged (JA-10 rotor, 8000 rpm, 10 min), bacterial sediment was resuspended in 4 ml P1 buffer and transferred in JA-20 tubes. After addition of 4 ml P2 the tubes were several times turned upside down to allow lysis of the cells and incubated for 5 min by RT. After addition of 4 ml of buffer P3 samples were immediately mixed, neutralised for 10 min on ice and centrifuged for 30 min by 12 rpm at 4°C in JA-20 rotor. The supernatant was transferred on the equilibrated with 10 ml QBT buffer Quagen column and bound DNA was washed twice with 10 ml QC buffer. The elution of DNA was performed with 5 ml QF buffer in 25 ml Correx-tubes and eluted DNA was precipitated with 0,7 ml isopropanol (SS34 rotor, 14000 rpm, 4°C). The DNA pellet was dissolved in 500µl distilled H_2O, transferred in a reaction tube and after addition of 0,3 vol 7 M NH_4Ac and 1 ml 100% ethanol precipitated for the second time for 30 min at –80°C. After centrifugation in

eppendorf centrifuge for 20 min by 14000 rpm at 4°C DNA was washed with 70% ethanol, dried in the air and dissolved in the appropriate volume of distilled H_2O. After determination of concentration and purity (see) concentration of DNA was adjusted to 1µg/µl and DNA was kept at –20°C.

2.16.2.3. Phenol/chloroform extraction of DNA

Extracton of DNA with a mixture of phenol/chloroform/isoamylalcohol (25:24:1) and chlorophorm/isoamylalcohol (24:1) is a standard method for removal of proteins from nuclein acid preparations. The purification was performed as follows: 1 volume of phenol mixture was added to DNA solution, well mixed and centrifuged for 5 min at 14000 rpm. The upper DNA containing phase was carefully taken out and in order to remove the rest of phenol was mixed with 1 volume of chlorophorm/isoamylalcohol mixture. After mixing and centrifugation at 14000 rpm for 5 min the upper phase was taken out.

2.16.2.4. Precipitation of DNA with ethanol

Precipitation and concentration of DNA was performed by adding to extracted DNA (see 3.10.3) 2-2,5 volumes of ethanol after addition of 0,3 volumes of 7M NH_4Ac. Sample was incubated for 30 min at –80°C. After pelleting by centrifugation for 20 min at 14000 rpm at 4°C, DNA was washed with 70% ethanol and the precipitate was dried in the air by RT.

2.16.2.5. Determination of concentration and purity of DNA

Purity and concentration of DNA was determined by measuring the absorbance by 260- and 280 nm against the buffer. One A_{260}-unit equals to concentration of 50µg/ml of double stranded DNA or RNA, 40 µg/ml of single stranded DNA or RNA and 31µg/ml of olygonucleotides. The ratio of absorbance by 260- and 280 nm is a relative measure of purity (F) of nucleic acid solution. Ratios less then 2.0 for DNA or 1,8 for RNA mean that the solution is contaminated with proteins, phenol or other organic contaminants.

2.16.2.6. Electrophoretic separation of DNA fragments in agarose gels

For analysis and purification of DNA fragments of different size horizontally oriented agarose gels were used (Sambrook *et al*., 1986). Agarose concentration of the gels was dependent on the size of DNA fragments to be separated:

Agarose concentration (% (w/v))	**Separation area (Kb)**
0.6	1-20
0.9	0.5-7
1.2	0.4-6
1.5	0.2-4
2.0	0.1-3

For preparation of the gel the appropriate agarose amount dissolved in 300 ml 1xTAE buffer was cooked in the microwave oven and after cooling it down ethidium bromide was added to reach the end concentration of 0,5µg per ml. Agarose solution was applied into a gel chamber with and combs with appropriate pocket size were introduced. After the gel was ready it was transferred into an electrophoresis chamber filled with 1xTAE buffer. The samples were mixed with 0,2 volume of ficoll-marker and transferred into gel pockets. Electrophoresis was performed by 4-5 V/cm. Etidium bromide is binding to DNA fragments and that makes possible visualisation of DNA by UV light. The detection limit of ethidium bromide stained gels is approximately 10ng of DNA per band. The agarose gels were developed on the UV-transilluminator and documented with the help of a video system.

2.16.2.7. Digestion of DNA with restriction nucleases

Restriction enzymes of type II recognize and cleave specific palindrome sequences of double stranded DNA. If the cleavage occurs between the opposite phosphodieste bonds, the resulting fragments have „blunt“ ends. If the cleavage is assymmetric, fragments have 3‘ or 5‘ „sticky“ ends. The activity of restriction endonucleases is expressed in Units (U). One unit of activity means that this amount of enzyme can totally digest 1µg of DNA standard (mainly Lambda-Phage DNA) in one hour. The restriction enzymes were used according to the protocols of the producers with appropriate buffers and by certain incubation temperature. For the preparative hydrolysis usually 4-5 units of enzyme per µg of DNA were taken and incubated at least for 2 hours.

3. Results

3.1. Optimisation and standardisation of the *in vitro* FGly generating assay

3.1.1. Construction and production of the in vitro FGly generating assay substrate (in cooperation with J.Fey)

As an *in vitro* substrate for detecting the activity of FGly generating machinery (FGM) we used ribosome-nascent chain complexes (RNCs) generated by *in vitro* translation of truncated mRNA encoding a polypeptide consisting of the heptapeptide MGLRMPD and the ASA residues 64-158 (see Fig.3.1). Since it was shown that FGly formation does not depend on the signal peptide and the assay could be performed in the absence of the membranes (Fey et al., 2001), the signal peptide was not present in the substrate. ASA residues 64-158 contained the sequence motif determining FGly formation and the heptapeptide MGLRMPD provided the initiator methionine and a tryptic cleavage site at position 4 followed by a second methionine. The latter allowed for [^{35}S]-methionine labelling of the tryptic peptide P2 carrying either FGly of cysteine in the position 69. This *in vitro* substrate was encoded by plasmid pMB2, which was constructed as follows. Using pTD17 (encoding ASA residues 19-200 with the natural methionines 85, 87 and 120 substituted by threonine (position 85) or leucine (position 87 and 120), see Dierks *et al*., 1997) as a template, the coding oligonucleotide CATGCCATGGGGCTGCGGATGCCGAC was added 5’ of ASA codon 64 by add-on PCR. The PCR product was cloned as an N*co*I/*Eco*RI fragment back into pTD17, thereby substituting the entire coding sequence.

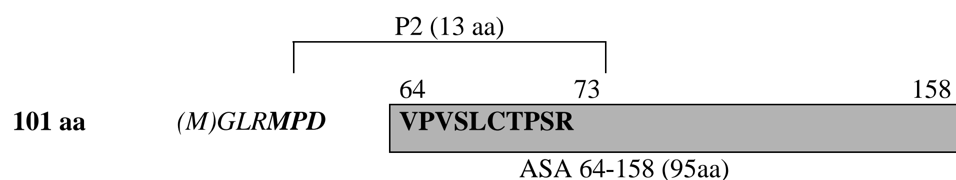


Fig.3.1. Substrate polypeptide used for *in vitro* FGly modification
The translation product shown here was generated by *in vitro* transcription and translation of the plasmid pMB2, which had been cleaved by *Alw*NI in the coding region 3’ of ASA codon 158. The product consists of the heptapeptide MGLRMPD (*italics*) and the ASA part comprising residues 64-158. The initiator methionine was cotranslationally removed by methionine aminopeptidase (see text). After tryptic cleavage, peptide 2 (P2) carried the only methionine and cysteine 69, as indicated. The positions of the labeled methionines were verified by radiosequencing of both intact translational product and its tryptic peptides.

PMB2 was cleaved by A*lw*N in the coding region 3' of ASA codon 158. Using this linearized DNA as a template, run-off transcripts lacking a stop codon were obtained by *in vitro* transcription with SP6 polymerase using (see Methods). The run-off transcripts were translated *in vitro* in a reticulocyte lysate translation system (see Methods) in the presence of [^{35}S]methionine. The RNCs obtained were separated from the translation mixture by sedimentation through a sucrose cushion in the presence of 500mM potassium acetate, yielding mRNA-programmed ribosomes with attached nascent chains. The RNCs were used as a substrate for the *in vitro* FGly generating assay. RNCs could tolerated storage at –80°C and after shock freezing in liquid nitrogen) with following storage at –80°C and up to 4 cycles cycles of freezing-thawing did not have any influence on the modification competence of the substrate.

3.1.2. Standard conditions for the *in vitro* FGly generating assay

The biochemical approach to identify and characterize FGM involves many chromatographic steps. To be able to estimate yields of enzyme activity for these purification steps and to characterize the FGly generating enzyme biochemically at the different levels of purity one needs to establish standard *in vitro* FGM assay conditions.

The optimised standard conditions were chosen as follows: aliquots of reticuloplasm or chromatographic fraction corresponding to 15-45 eq (for determination of equivalents see Methods, paragraph 2.15.2) of microsomal membranes were incubated in a final volume of 100µl with 5 µl of salt-washed RNCs (≈30000 dpm, estimated to correspond to ≈15fmol of polypeptide substrate) for 20 min at 37°C in the presence of 1,5mM puromycin in OG buffer. Amount and volume of enzyme from the purified fractions and, accordingly, the volume of OG buffer used to fill up the assay volume up to 100µl differed from experiment to experiment, but the substrate and puromycine concentration, assay volume, temperature and incubation time were kept constant.

Incubation was stopped by addition of preheated (80°C) carboxymethylation buffer containing 6M guanidine hydrochloride. Samples were subjected to reductive carboxymethylation and trypric digestion (see Methods).

Tryptic cleavage of the samples led to a single ^{35}S-labeled peptide of 13 residues comprising the MPD tripeptide followed by the ASA sequence 64-73 (Fig.3.1). The MGLR tetra peptide was not detected in the tryptic digest, indicating that the initiator methionine had been removed by methionine aminopeptidase present in the translation system. The tryptic peptides

were analysed by RP-HPLC on a C8 column that allows to separate modified and unmodified forms of peptide 2 (ASA residues 64-73 preceded by the tripeptide MPD) containing FGly or carboxymethylcysteine, respectively, at position 69 (Fig.3.2). The presence of FGly in the early eluting form but not in the later eluting form of peptide 2 was verified by reaction with dinitrophenylhydrazine (DNPH). Only the former could be converted into a hydrazone derivative (up to 95% efficiency, Fig.3.3). Hydrazon formation depends on the presence of an aldehyde group and increases the peptide hydrophobicity, which is utilized to separate the hydrazone derivative from unreacted peptide 2 and/or contaminating radioactivity that coeluted with the modified peptide from the C8 column (Dierks et al.,1997, 1998, 1999).

3.1.3. Quantitative estimation of FGly formation

Quantitative estimation of FGly formed during the assay was performed as follows: assuming that amount of substrate ASA polypeptide in 5µl RNCs (≈30000 dpm) corresponds to 100% we calculated percentage of the first, FGly form of the peptide 2 peak (Fig 3.2) in the sum of the two radioactive peaks. Fractions containing this peak were subjected to incubation with DNPH, resulting in formation of the hydrazon derivative of the FGly form of the peptide 2. After RP-HPLC separation of peptide 2 from its hydrazone derivative the percentage of derivative form (late eluting peak, 5min, Fig.3.3) in the mixture was calculated. Total percentage of the FGly form of the peptide 2 was then calculated on the basis of these two numbers (see example in Table 3.1).

To compare the activity of reticuloplasm and purified enzyme we used the unit „%FGly per equivalent" („%/eq"). 1% per equivalent corresponded to FGly formation in 1% substrate ASA polypeptide present in the assay mixture by 1 microsomal equivalent during 20 min incubation time under standard assay conditions. Activity expressed in % per equivalent could be calculated by dividing the percentage of FGly form of peptide 2 through total number of equivalents used in the assay (see example in Table 3.1):

$$\frac{\text{\% of FGly form of peptide2}}{\text{Total equivalents in the assay}} = \text{\% FGly/equivalent}$$

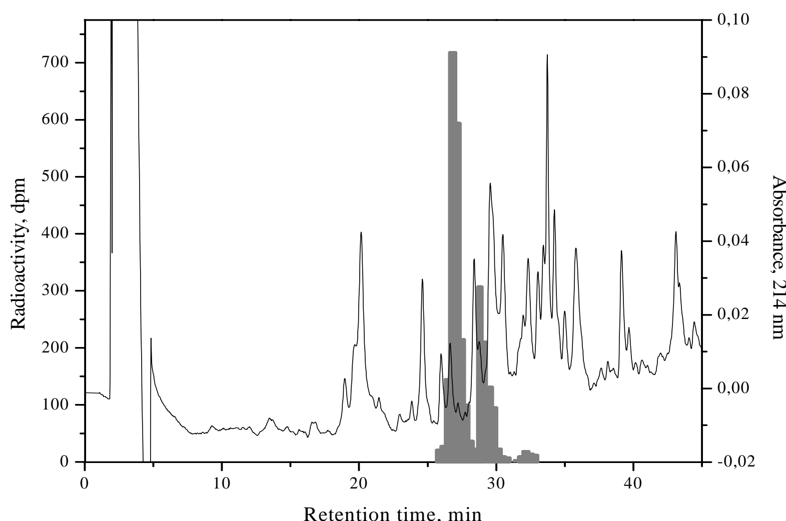


Fig.3.2. *In vitro* modification of peptide 2 of ASA.
The mixture of modified and unmodified form of peptide 2 was separated after carboxymethylation and tryptic digestion on a RP-HPLC C8 column. The labeled peptides were localized by liquid scintillation counting of the chromatographic fractions (see histogram). The early eluting radioactive peak (71.8% of the sum first+second radioactive peaks) corresponds to the FGly form of peptide 2, the later eluting peak (28.2%) to the cystein form of peptide 2, which was confirmed by radiosequencing (see Dierks *et al*., 1997,1998,1999) and by reaction with DNPH (Fig.3.3).

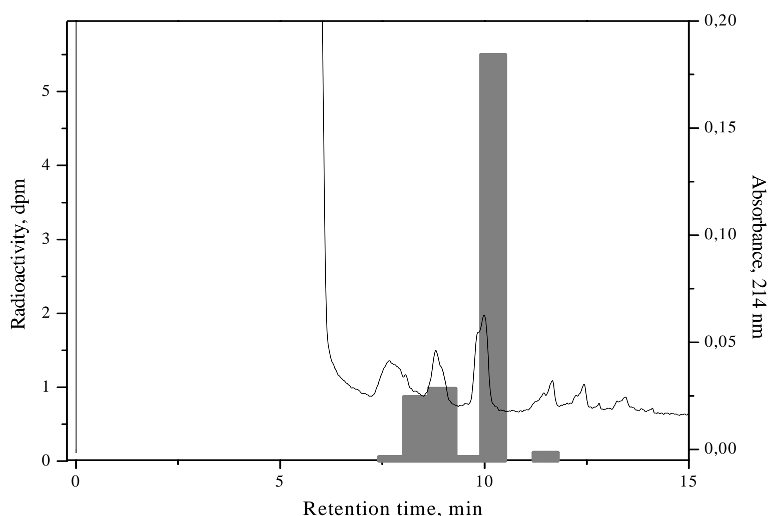


Fig.3.3. Generation of an aldehyde group in peptide 2 of the substrate.
The [^{35}S]methionine labeled peptide 2 was assayed for the presence of an aldehyde group by reaction with DNP-hydrazine. After incubation of the peptide with the reagent, P2 and its DNP-hydrazone derivative were separated by RP-HPLC on a C18 column. The modification efficiency (hydrazone derivative as percentage of P2 plus derivative) was calculated on the basis of the radioactivity recovered in the corresponding fractions (histogram). Modification efficiency in this case was 82% (percentage of the second peak calculated from the total radioactivity). For more details see Dierks *et al*., 1997,1999.

Table 3.1. Example of quantitative estimation of FGM activity of reticuloplasm. Table data are based on results shown in Fig.3.2 and Fig.3.3.

Sample	**Total equivalents**	**Early eluting radioactive peak after RP-HPLC separation (Fig.3.2) (% of the sum early+late peaks)**	**Hydrazon derivative (Fig.3.3) (% of total radioactivit y)**	**Peptide 2 containing FGly (%)**	**Estimated activity (%FGly/total equivalents, % FGly per equivalent)**
Reticu-loplasm, 10µl (3 eq per µl)	30	71.8	82	58.87	1.96

By expressing activity of reticuloplasm and later the activity of purified chromatographic fractions in % per equivalent we were able to compare activity of FGly generating enzyme in different preparations of reticuloplasm, estimate the activity loss, enrichment and recovery after purification steps as compared to the activity in the starting material

3.2. Bovine rough microsomes as a source of FGM activity

The investigations of FGly formation and properties of the eukaryotic FGly generating machinery (FGM) were performed using dog pancreas rough microsomes or their detergent extract as the source for the FGM activity (Dierks *et al*.,1997,1998,1999; Fey *et al*., 2001). For ethical and technical reasons the dog microsomes are not easy to obtain and due to the small size of a dog pancreas, the microsomes could not be used as the protein source for purification of FGM on a big scale. On the contrary, bovine tissues are available from the local slaughterhouse in unlimited amount. Thus, it was tested whether bovine tissues could be used as the source for FGM. Rough microsomes were prepared from bovine liver, testis, adrenal glands and pancreas (see Methods). The luminal components of the microsomes (reticuloplasm) were extracted with the detergent DBC under conditions that avoid solubilisation of the membranes (see Methods) and tested for FGM activity. As can be seen from Table 3.2, microsomes from different tissues differed in terms of protein concentration and FGM activity.

Table 3.2. Comparison of FGM activity in different tissues. For determination of equivalents see Methods, paragraph 2.15.2; FGly % per equivalent units are determined above.

Tissue	Microsomes (equivalents per µl)	FGM activity (% per equivalent)	FGM activity (% per g wet tissue)
Dog pancreas	1	6.7	2680
Cow pancreas	3	1.5	8010
Cow testis	0.2	13	572
Cow adrenal glands	0.3	Could not be determined	Could not be determined
Cow liver	1.1	2.9	2394

In case of reticuloplasm obtained from adrenal gland tissue, activity of FGly generating enzyme could not be determined due to the high activity of proteases, which led to substrate destruction. In some preparations of pancreas microsomes we also encountered very high levels of proteases activity. Thus, these preparations could not be used for further purification of FGly generating enzyme due to destruction of the substrate. High levels of protease activity could possibly be explained by partial contamination of the membrane fraction by cytosolic components during microsomal preparations. This problem could be eliminated by doubling concentration of protease inhibitors cocktail present in the homogenisation buffer used for preparation of microsomes (see Methods).

After testing rough ER microsomes from different tissues we have chosen cow pancreas as a starting protein source for purification of FGM. Although cow pancreas microsomes had up to 4-fold lower specific modification activity as compared with dog pancreas microsomes, they showed the highest FGM activity per gram wet tissue as compared to dog microsomes and microsomes from other bovine tissues, could be obtained in unlimited amounts and did not have high levels of protease activity. Bovine testis could not be used because due to low levels of FGly generating activity per gram wet tissue one should use much more testis tissue per preparation then that of pancreas and capacities of the available centrifuges allowed us to process only 50-100g wet tissue per preparation.

3.3. Kinetic and biochemical characterisation of FGly generating enzyme (in cooperation with J.Fey)

For reliable analysis of FGly modification in dependence of different parameters and for definition of the standard assay conditions we had to characterise the FGly generating enzyme kinetically and biochemically. Experiments were done to estimate time, dose and temperature dependence of FGly formation *in vitro*, in addition the pH optimum and the inhibition of FGly formation by ASA peptide 65-80, and the sensitivity of FGly generating enzyme to salts.

3.3.1. Time dependence of FGly formation

FGly formation by 45 eq of reticuloplasm was assayed at different incubation times (0-60 min) (Fig. 3.4.*A*). Under the standard *in vitro* assay conditions the FGM shows slow kinetics (approximately 1% FGly per equivalent in 20 min). For further experiments an incubation time of 20 min was chosen. The reaction shows linear kinetics until 40-50% of the substrate is turned over. At longer incubation times the time curve levels off (Fig. 3.4.*A, inset*).

3.3.2. Dose dependence in FGly generating assay

Different amounts of reticuloplasm (1-100 eq) were assayed for FGly formation during 20 min incubation. As can be seen from Fig. 3.4.*B*, FGly modification is linear from 0 up to almost 60 eq (0 to 40µl reticuloplasm) and then levels off, what could be explained by substrate limitation when more than 50% of the substrate is converted to the FGly product (compare to Fig. 3.4.*A*).

3.3.3. pH optimum of FGly activity

FGly formation was assayed using 20µl (30eq) reticuloplasm prepared in OG buffer (see Methods) with 10µm HEPES-KOH (pH 8.0). Immediately before addition of RNCs and puromycin the assayed reticuloplasm was diluted to 87,5µl with extraction buffer comprising 50mM HEPES/CAPS or HEPES/Tris (pH 6,7,8,9,10,11,12, or 13, respectively). After 20 min, incubation was stopped with preheated RCM buffer and the assay mixture was analysed for the presence of the FGly peptide. In Fig. 3.4.E the effective pH, measured after mixing the assay components is shown. Resulting pH optimum was highly alkaline (pH 10-10.5). At pH 7, activity was about 50% and at pH 11 about 30% of optimum activity. Thus, FGly formation can be assayed at a broad pH range (7-11).

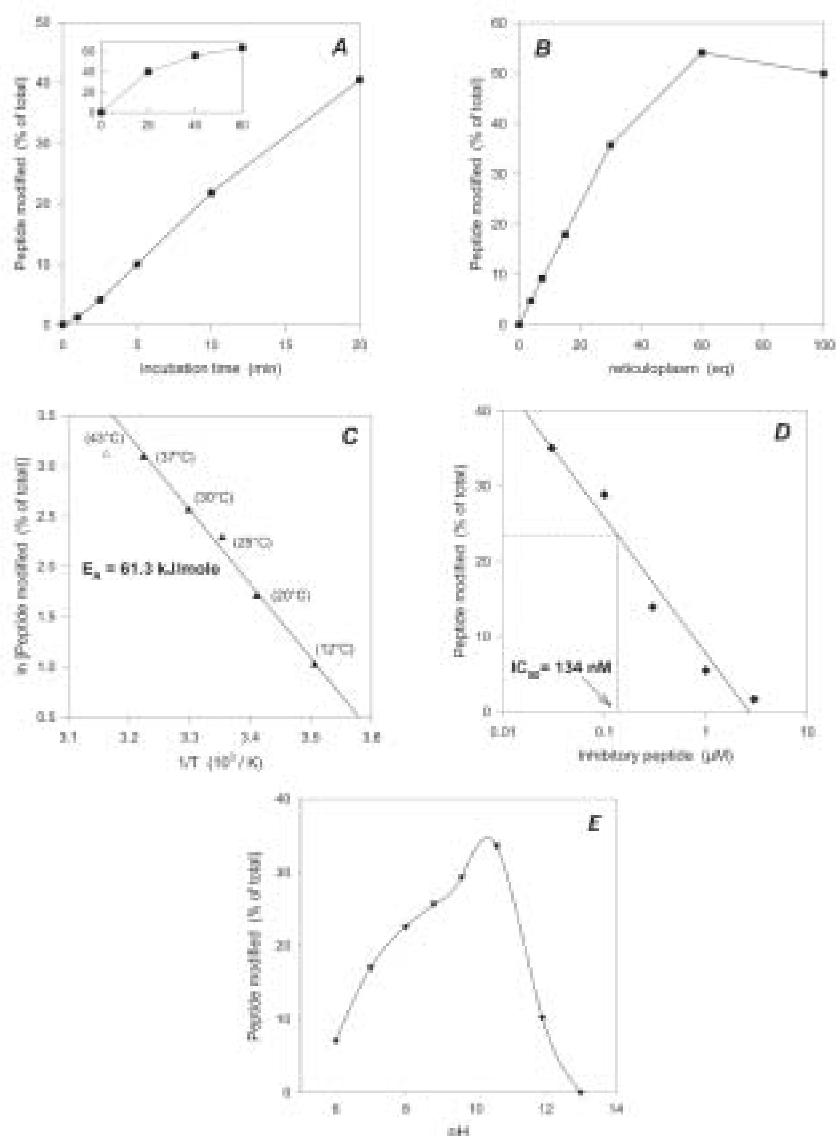


Fig. 3.4. Kinetic characterisation of FGly modification by reticuloplasm. Bovine pancreas reticuloplasm was assayed under standard assay conditions. *A*, time dependence of FGly formation. *B*, dose dependence of FGl formation. *C*, temperature dependence of FGly . The results are given in Arrhenius plot. Except for for the 43°C value (open triangle) all data (closed triangles) could be fitted by a straight line. From the slope of the line an activation energy (E_A) was calculated. *D*, inhibition of FGly formation by ASA peptide 65-80. FGly modification was assayed in the presence of different concentrations (0-3µM) of ASA peptide 65-80 (see text). The data are shown in a semi-logariphmic plot, from which an IC_{50} value was calculated. In the abcence of the inhibitory peptide, 46,9% of total substrate molecules were modified. *E*, pH dependence of FGly formation.

3.3.4. pH stability of FGly generating enzyme

The pH optimum studies showed that FGly is fastest at pH 10-10.5. Decreasing pH results in the decrease of the FGM activity. Since in some chromatographic procedures (cation exchange chromatography, affinity chromatography) low pH conditions are necessary for

efficient binding or elution of the bound proteins from the chromatographic matrices, we performed a test for estimating the stability of the FGM after treatment at various pH-values. 135 eq reticuloplasm were passed over a gel filtration column (Fast Desalting PC 3.2/10) to exchange OG buffer to 50mM Tris pH 9.0, pH10.5 or to 50mM glycine pH 2.8 or pH 3.5. As a control 135 eq reticuloplasm were passed over Fast Desalting column in OG buffer to serve as a control. Immediately after exchanging the buffer, the protein peak fractions were neutralized to pH 7.5 with Tris or glycine buffers and the samples were incubated with RNCs and puromycin for estimation of FGM activity. After treatment with low pH (2.8 and 3.5) activity of FGly enzyme was totally inhibited and could not be recovered after neutralisation. Thus, the inhibition of the enzyme activity by low pH was irreversible. After treatment with high pH buffers (pH 9.0 and 10.5) the activity was unaffected as compared to the control.

3.3.5. Temperature dependence of FGly formation

30 eq reticuloplasm were assayed for FGly formation at different incubation temperatures (12-43°C) during incubation with RNCs and puromycin. The results (Fig. 3.4.*C*) are given in an Arrhenius plot. Up to a maximum of 37°C the modification showed a typical temperature dependence, whereas at 43°C we observed enzyme inactivation. From the slope of the graph an activation energy of the FGM (E_A) of 61,3 kJ/mol was calculated, which is well within the range expected for an enzyme-mediated reaction.

3.3.6. Inhibition of the FGly generating enzyme activity by ASA peptide 65-80

30 eq reticuloplasm were assayed for FGM activity in the presence of different concentrations (0-3µM) of ASA peptide 65-80, comprising the modification motif. The activity of the FGM was inhibited up to 100% with increasing concentrations of the ASA peptide. Half-maximum inhibition was observed at a concentration of 134 nM (see Fig.3.4.*D*). This IC_{50} value may be taken as a figure of the enzyme's affinity constant. A true K_m value could not be determined by saturation studies, because of substrate limitation (approximately 15 fmol of *in vitro* synthesized polypeptides). At this low substrate concentration binding of the substrate to the enzyme's catalytic centre (and not catalysis) could be the rate-limiting step.

As shown earlier (Dierks *et al.*,1999), the cysteine 69, arginine 71 and proline 73 are the three critical residues in the ASA sequence which are supposedly recognised by FGly generating enzyme. Therefore, we also assayed whether ASA peptides 65-80 with mutated critical residues would inhibit the FGM activity. Two peptides were particularly important: ASA 65-

80 with cysteine 69 substituted by serine and ASA 65-80 with scrambled positions of the three critical residues, namely C69P, P71R and R73C scrambled. The ASA C69S peptide totally inhibited FGM activity in the reticuloplasm by 50% at the concentration 1μM. At the concentration 5 μM the inhibition was almost 80%. The ASA peptide with scrambled sequence did not have any inhibitory effect.

3.3.7. Sensitivity of the FGM to salts

Since many chromatographic procedures include steps with high salt buffers, it was important to test the sensitivity of the FGM to different salts. We have chosen those salts which later were used for chromatographic procedures: NaCl (ion exchange chromatography), KSCN (affinity chromatography) and $(NH_4)_2SO_4$ (hydrophobic interaction chromatography).

To test the sensitivity of FGly generating enzyme to NaCl 30 eq reticuloplasm were incubated for 20 min with RNCs and puromycin in the presence of 1,5 M NaCl. Activity recovery was 50% of the control. After removal of the salt by dialysis 100% of starting activity was recovered.

For testing the sensitivity to KSCN 30 eq reticuloplasm were incubated with 2M KSCN for 30min. Then the sample was subjected to buffer exchange against OG buffer on a PD10 gel filtration column (see Methods). The sample containing 2M KSCN during incubation with RNCs and puromycin showed complete inhibition of FGM activity. After buffer exchange by gel filtration 50% of the untreated control sample were recovered.

When 30 eq reticuloplasm were incubated with 2M $(NH_4)_2SO_4$ for 40 min at room temperature (conditions of HIC chromatographic run) and then subjected to desalting and buffer exchange against OG buffer in VivaSpin concentrators, the activity of reticuloplasm was 58% as compared to the untreated sample.

3.4. Single chromatographic protocols for separation of the reticuloplasm proteins and partial purification of FGM activity

Having established the standard assay conditions and having characterised basic biochemical properties of the FGly generating machinery we developed several single chromatographic protocols for separation of the reticulolasm proteins and partial purification of FGM activity.

3.4.1. Anion exchange chromatography

3.4.1.1. MonoQ® PC 1.6/5 (0.1ml) and MonoQ® HR 5/5 (1ml) columns for SMART system, separation of reticuloplasm proteins at pH 8.0, 9.0 and 10.0

MonoQ® PC 1.6/5 (0.1ml) column for SMART system is a strong anion exchanger that allows separation of proteins according to their surface charge on the analytical scale (up to 3 mg total protein). Proteins were bound to the column at low salt concentration and eluted then with a salt gradient from 0 to 0,75 M of NaCl. For testing its chromatographic behaviour on the MonoQ column 750 eq reticuloplasm (250µl microsomes, corresponding to approximately 0,75mg total protein) were concentrated in a Vivaspin concentrator to 150µl and then diluted with OG buffer containing no KAc. Thus the salt concentration in the sample was reduced from 150mM to approximately 50 mM, to ensure binding of the proteins to the column. The sample was applied on the column through a 500µl loop. Flow through and eluting proteins were collected in the fractions of 100 µl. 15µl of the fractions were analysed by SDS page (Fig. 3.5) and 85 µl were incubated with RNCs and puromycin and tested for FGM activity. The assay results showed that FGM was relatively weakly bound to the column as all the activity was recovered in the fractions at the beginning of the gradient (see Fig.3.6, *A*) About 50% of the loaded reticuloplasm activity was recovered in these fractions.

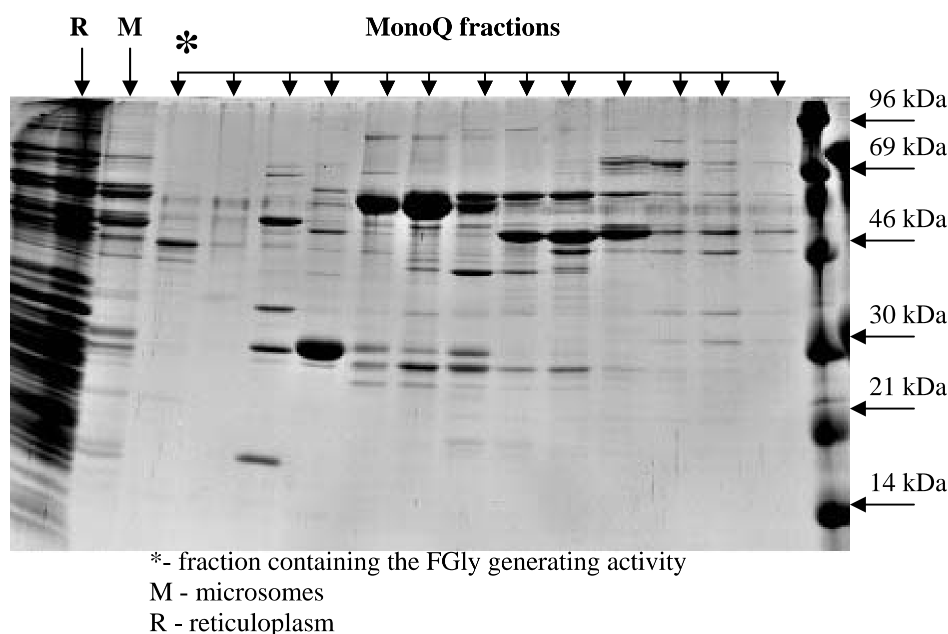


Fig. 3.5. Separation of reticulolasm proteins on MonoQ® PC 1.6/5 anion exchange column for Smart system at pH 8.0. Proteins of 250µl reticuloplasm were separated on MonoQ anion exchange column. Fractions of 100µl were collected and 15 µl of each fraction were applied on a 17,5% acrylamide SDS gel and stained with Coomassie blue.

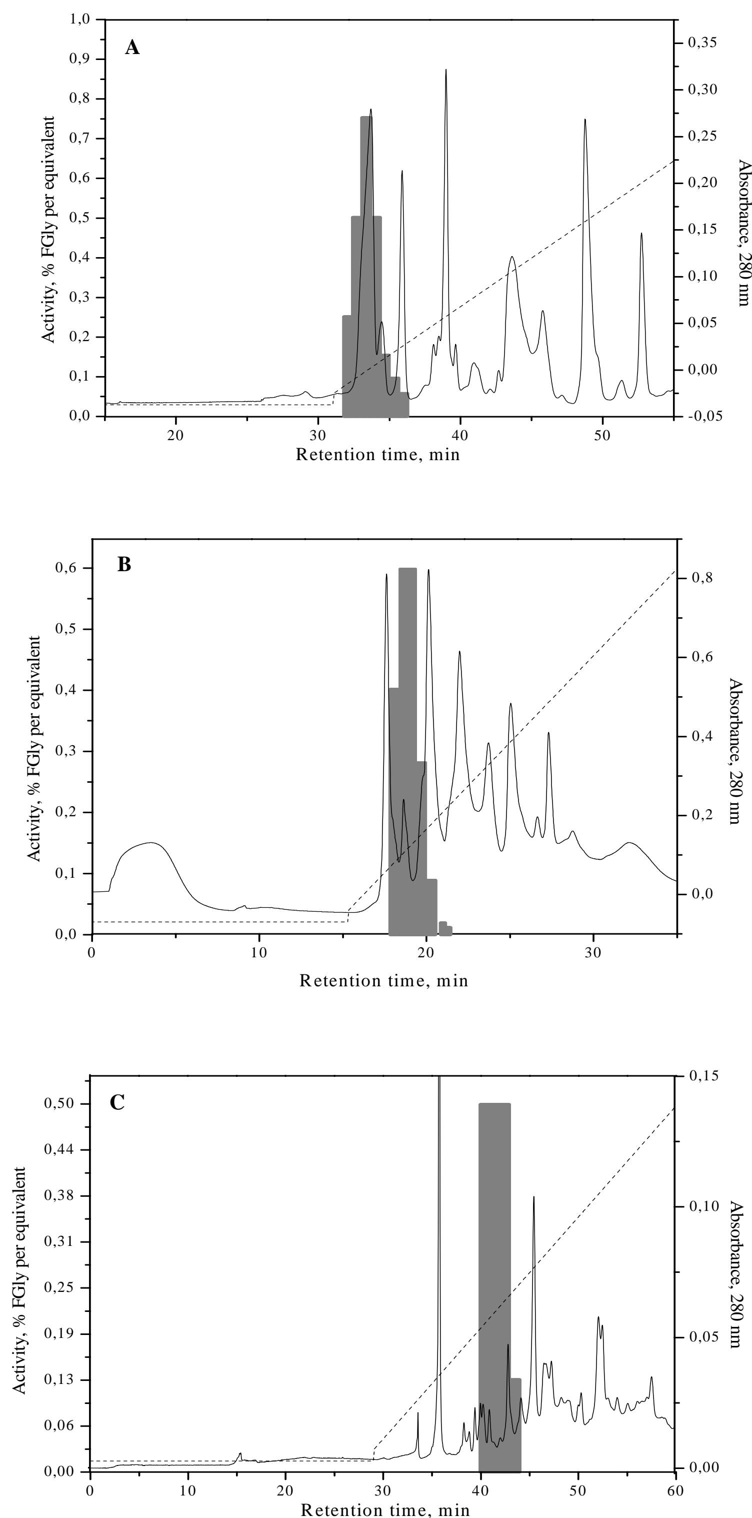


Fig.3.7. Separation of reticuloplasm proteins on MonoQ PC 1.6/5 (MonoQ HR 5/5) anion exchange column at pH 8.0, 9.0 and 10.0. Reticuloplasm proteins were separated on the MonoQ anion exchange column at different pH values. The dashed line shows the conductivity measured directly after passing the flow cell of the SMART system.FGM activity elution profile is indicated in grey bars. At pH 8.0 (A) FGM activity elutes in the very beginning of the salt gradient (at approximately 75mM NaCl). Increasing pH of the buffer solutions to pH 9.0 (B) and pH 10.0 (C) ensures stronger binding of FGM activity to the column matrix (elution with 100-150mM NaCl at pH 9.0 and 200-250mM NaCl at pH 10.0).

For testing the MonoQ® HR 5/5 (1ml) column, 15000 eq reticuloplasm (corresponding to 5ml microsomes or 15 mg total protein) were reduced in salt concentration in the same way as for MonoQ® PC 1.6/5 column and applied through a 10ml loop. Bound proteins were eluted with a gradient from 0 to 0,75M NaCl in 20 column volumes. Activity and protein elution profile were more or less the same as for the small scale MonoQ® PC 1.6/5 (0.1ml) column (not shown).
all the activity was eluted in the very beginning of the salt gradient and coeluted with a big protein peak (Fig. 3.6), we tried to increase the strength of protein binding to the anion exchanger by increasing the pH of the binding and elution buffers. At higher pH the protein should be more negatively charged, which ensures stronger binding to the positively charged column matrix. Although for some proteins a high pH can lead to inactivation, the FGM has an unusual pH optimum of pH 10-10.5 which allows increasing the pH of the chromatographic buffers without inactivation. We separated reticuloplasm proteins on the MonoQ HR 5/5 column at pH 9.0 (Fig.3.6, *B*) and pH 10.0 (Fig.3.6, *C*) As could be seen from the protein and activity elution profiles, proteins were bound stronger to the anion exchange matrix at high pH. The recovery of FGM activity after runs performed at pH 9.0 and 10.0 was estimated to be the same as for the runs performed at pH 8.0.

3.4.1.2. MonoQ® HR 10/10 (8ml) column for SMART system

The MonoQ® HR 10/10 (8ml) column for SMART system allows separation of proteins on the preparative scale (up to 400mg total protein). We could separate up to 120000 eq reticuloplasm (corresponding to 40ml microsomes, 120 mg total protein) in a single run on this column. The preparation of the sample was similar as for MonoQ PC 1.6/5 and MonoQ HR 5/5 columns. The reticuloplasm was concentrated to 10-15ml in Vivaspin concentrators and then diluted to 50ml with 20mM Tris pH 8.0 (MonoQ buffer A). To remove protein aggregates, the sample was passed through 0.45 or 0.22µm filters before being applied on the column. Application of the sample was performed through a 50ml super loop from Pharmacia. Bound proteins were eluted with a 10 column volumes gradient of NaCl from 0 to 0.75M. Flow through and gradient fractions were tested for FGM activity.
As show in Fig.3.7, the protein elution profile of MonoQ® HR 10/10 column was similar to that of MonoQ PC 1.6/5 and MonoQ HR 5/5 columns. The activity elution profile, though, differed, as the activity peak eluted from MonoQ® HR 10/10 earlier in the gradient and was

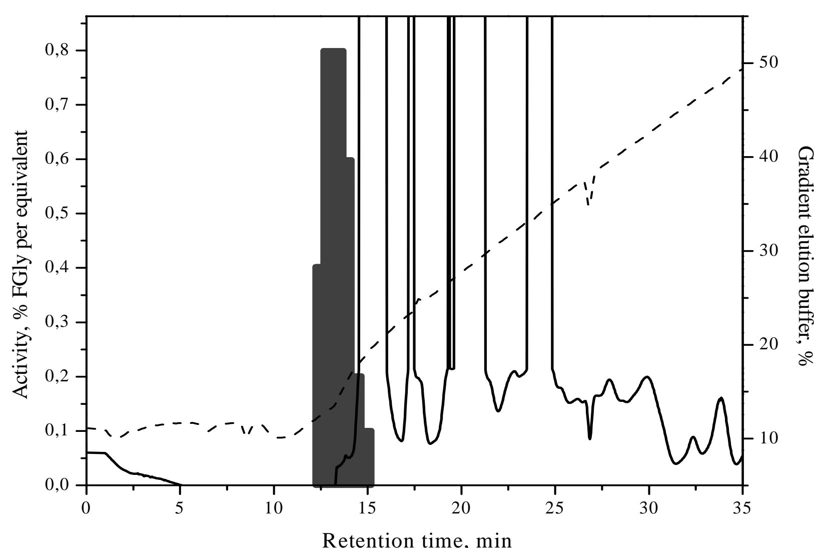


Fig. 3.7. Separation of reticulolasm proteins on MonoQ HR 10/10 anion exchange column at pH 8.0. FGM activity (shown in grey bars) is eluting from the column at the very beginning of salt gradient (approximately 50-75mM NaCl), preceding the first big protein peak. The dashed line shows the conductivity measured directly after passing the flow cell of the SMART system. Active fractions comprise approximately 1-3% of total reticuloplasm proteins.

just preceding the big protein peak with which it coeluted from MonoQ PC 1.6/5 and MonoQ HR 5/5 columns. Activity recovery from MonoQ® HR 10/10 was estimated to 40-60%.

This activity elution profile and the good activity recovery made the MonoQ® HR 10/10 column very suitable for purification of FGM activity on a big scale. We took advantage of the fact that FGM activity eluted from this column very early in the gradient, just preceding the big protein peak, what made the purification of FGM activity on MonoQ® HR 10/10 several fold more efficient than on MonoQ PC 1.6/5 and MonoQ HR 5/5 columns.

3.4.2. Cation exchange chromatography on MonoS® PC 1.6/5 (0.1ml) for SMART system

The MonoS® PC 1.6/5 (0.1ml) column for SMART system is a strong cation exchanger that allows separation of proteins according to their surface charge on the analytical scale (up to 3 mg total protein). Proteins are bound to the column at low salt concentration and eluted then with a gradient from 0 to 0,75 M of NaCl. For testing its chromatographic behaviour on the column, 750 eq of reticuloplasm (250µl reticuloplasm, corresponding to approximately 0,75mg total protein) were concentrated in a Vivaspin concentrator to 150µl and then diluted with MonoS buffer A (20mM MES pH 6.0). Thus, the salt concentration in the sample was reduced from 150mM to approximately 50 mM and the pH was also adjusted to ensure binding of the proteins to the column. The sample was applied on the column through a 500µl

loop. Flow through and eluting proteins were collected in fractions of 100 µl. 85 µl of the fractions were subjected to buffer exchange in Vivaspin concentrators followed by incubation with RNCs and puromycin to test for FGM activity. The activity assay results showed that FGly generating enzyme did not bind to the column, as all activity was recovered in the flow through (see Fig.3.8) Activity recovery was estimated to 10% as compared to that of the loaded reticuloplasm.

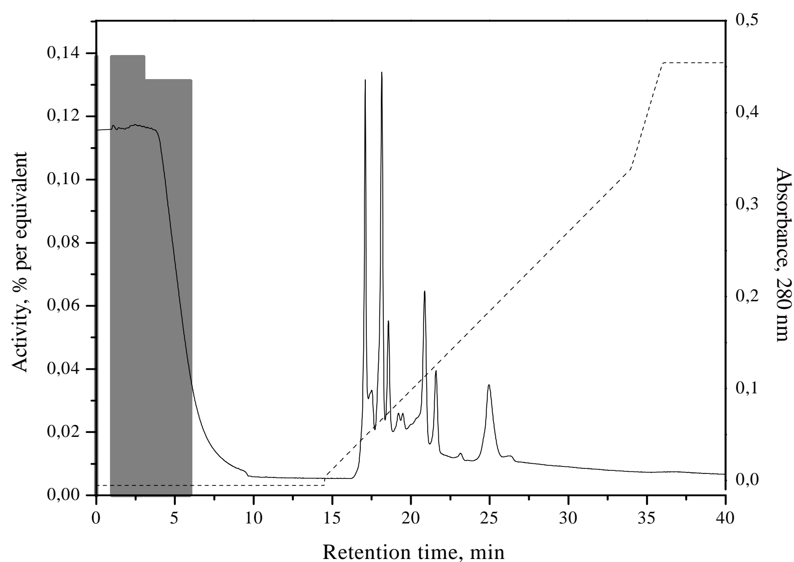


Fig. 3.8. Separation of reticuloplasm proteins on MonoS® PC 1.6/5 cation exchange column for SMART system at pH 6.0. The dashed line indicates the gradient of the elution buffer. At pH 6.0 no FGly generating activity is bound to the column matrix and elutes in the flowthrough (activity profile is shown in grey bars).

A possible way to increase the strength of protein binding to the cation exchanger column is to decrease the pH of the buffer solutions. At lower pH the protein becomes protonated, ensuring stronger binding to the negatively charged column matrix. We have performed separation of reticuloplasm proteins on the MonoS column at the pH 5.5. At these conditions FGM activity was strongly bound to the column matrix as we could detect the activity in the fractions corresponding to the end of the gradient (see Fig.3.9), but the recovery of activity was very low (1% of the load). pH optimum and pH stability studies (see 3.3.3 and 3.3.4) showed that at pH-values lower then 6 the activity of FGly generating enzyme is severely decreased and that this inhibition by low pH is irreversible. Thus, we were not able to estimate the true recovery of the enzyme after purification on the MonoS column, since low activity values could mean both loss of protein and inactivation due to low pH values.

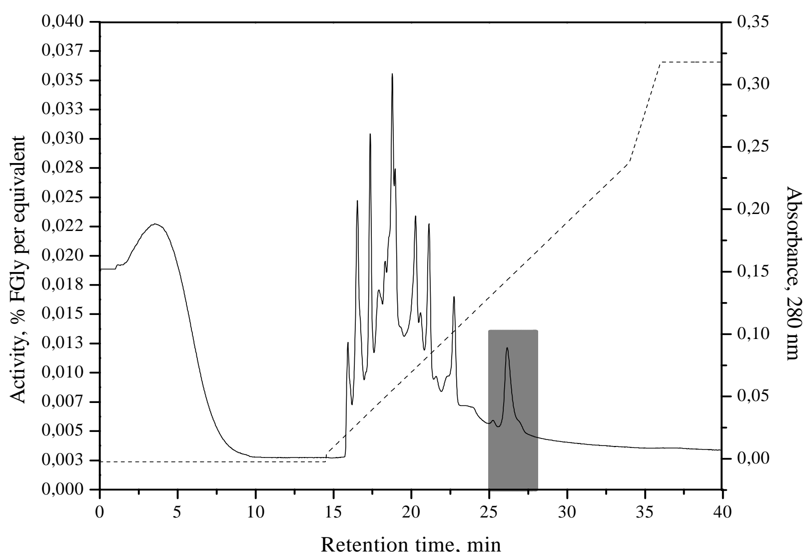


Fig. 3.9. Separation of reticuloplasm proteins on MonoS® PC 1.6/5 cation exchange column for SMART system at pH 5.5. At pH 5.5 FGly generating activity bound to the column matrix and elutes late in the gradient (activity profile is shown in grey bars). The dashed line shows gradient of the elution buffer. Activity recovery, though, was estimated only 1% of the starting material.

3.4.3. Hydrophobic interactions chromatography on the Phenyl Superose® PC 1.6/5 (0.1ml) column for SMART system

We established purification of reticuloplasm proteins based on their hydrophobicity using Phenyl Superose® PC 1.6/5 (0.1ml) column for SMART system (analytical purification, up to 3mg total protein).

600 eq reticuloplasm (approximately 0,6mg total protein) were subjected to concentration in the Vivaspin concentrator with buffer exchange to 50mM NaPi pH 7.0, 2M $(NH_4)_2SO_4$. The sample was applied on the column equilibrated with the same buffer through a 500µl loop. Elution of the bound proteins was performed with a 20 column volumes linear decreasing gradient of salt to from 100% to 0% loading buffer. Fractions of 100µl were collected. 50µl of each fraction were subjected to buffer exchange in the Vivaspin concentrator against OG buffer and then the fractions were tested for FGM activity. FGM activity coeluted with the big protein peak at the end of the gradient and was distributed over many fractions (Fig.3.10). Total recovery of activity was estimated to 20%.

A test of sensitivity of FGly generating enzyme to salts (see 3.3.6) showed that treatment with 2 M $(NH_4)_2SO_4$ led to around 40% irreversible activity inhibition. This could partly explain the low recovery values after purification on the Phenyl Superose column. At the same time

the purification itself was not very efficient as FGM activity coeluted with the main protein peak. The fact that activity eluted from the column in the end of the gradient indicates strong binding of the FGM to the column matrix. We therefore reduced the starting concentration of $(NH_4)_2SO_4$ to 1,5 M in order to increase activity recovery and also to try to separate the activity peak from the main protein peak. Indeed, the activity recovery increased to 50% and the reduced salt concentration led to weaker binding of FGM activity to the column matrix. Thus, the activity no longer coeluted with the main protein peak and was better separated from the majority of other proteins (Fig.3.11). Aliquots of the fractions were analysed by SDS-PAGE (Fig. 3.12.)

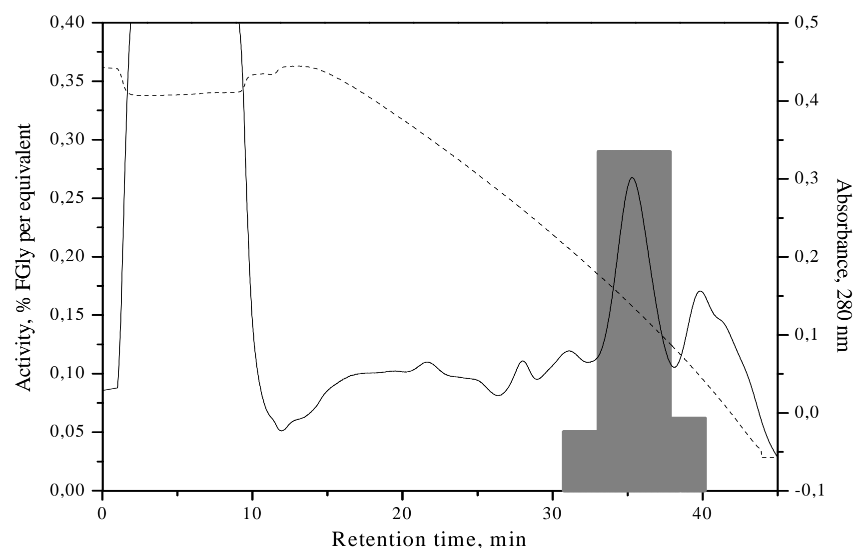


Fig. 3.10. Separation of reticuloplasm proteins on Phenyl Superose PC 1.6/5 hydrophobic interaction column at a starting salt concentration of 2M $(NH_4)_2SO_4$. Bound proteins were eluted from the column with decreasing gradient of salt. The activity elution profile is shown in grey bars. The dashed line shows the conductivity measured directly after passing the flow cell of the SMART system.

We have chosen separation of the reticuloplasm proteins on the Phenyl Sepharose column with 1,5M $(NH_4)_2SO_4$ as the optimal conditions for partial purification of FGM activity with maximal possible recovery of activity.

3.4.4. Gel filtration chromatography on Superdex® 200 PC 3.2/30 (2.4ml) column for SMART system

50µl (150eq) reticuloplasm were applied on a Superdex® 200 PC 3.2/30 gel filtration column equilibrated with OG buffer and eluted with 1 column volume of the same buffer at a flow rate of 40µl per min. Fractions of 100µl were collected. 77,5µl of each fraction was incubated

with RNCs and puromycin and tested for FGM activity. The FGM activity peak corresponded to protein fractions eluting with approximately 0,7 column volumes (Fig.3.13 and Fig.3.14). From a calibration of the column using molecular weight standards the size of FGly generating enzyme could be estimated to approximately 50 to 70 kDa.
The activity test showed that total recovery of FGM activity after passing reticuloplasm over gel filtration column was about 70%. This high recovery made the Superdex 200 column a good candidate for purifying the FGly generating enzyme on the analytical scale. However, the low sample application volume (maximal 50µl) and low capacity of the column made it unsuitable for a big scale purification.

3.4.5. ConA Sepharose chromatography

The FGM is localised to the ER lumen (Fey *et al*., 2001). Many of ER lumen resident proteins and proteins synthesized in the rough ER are glycosylated by the addition of a common *N*-linked oligosaccharide, composed of *N*-acetylglycosamine, mannose and glucose residues. To test whether the FGly generating enzyme is such a glycosylated protein we performed chromatography on Concanavalin A Sepharose (Con A Sepharose).
Concanavalin A is a lectin which reversibly binds molecules containing two non-substituted or C-2 substituted α-mannopyranosyl residues in one molecule. If FGly generating enzyme would carry *N*-linked oligosaccharides of this type, it would bind to the column matrix and thus would be separated from the non-glycosylated part of the reticuloplasm proteins and proteins carrying sugars chains of other types. In the opposite case it would not bind to the column matrix, would be then recovered in the flow through and thus would be separated from glycosylated proteins. In both cases passing reticuloplasm proteins over ConA Sepharose would be a good purification step, providing the recovery of FGM activity is good.
According to the protocol provided by the producer of ConA Sepharose (Amersham Pharmacia), binding and elution from the column matrix should be performed in the presence of 0,5M KCl. Since high salt concentrations have inhibitory effect on FGM activity and could be an obstacle for further purification steps, we performed chromatography on ConA Sepharose at both conditions (with and without salt).
225 eq reticuloplasm was subjected to buffer exchange to binding buffer with or without salt (see Methods) and applied on 100µl of ConA Sepharose equilibrated with the corresponding buffer. Binding of the proteins was performed at 4°C for 30 min. After collecting the flow through, the columns were washed with the binding buffer and eluted with buffer containing

0,5M methylmannopyranoside (with and without salt, respectively). The flowthrough and the eluates from both columns (see Fig.3.15) were tested for FGM activity.
The tests showed that FGly generating enzyme was completely bound to the column matrix in both cases, since the flow through from both columns was devoid of FGM activity. In the eluates the FGM activity was recovered with an efficiency of 22% (with salt) and 33% (without salt). Activity in the eluates was measured directly without buffer exchange. That could explain why in the case of the eluate with salt the activity recovery was lower.
Binding of FGM activity to ConA matrix shows that FGly generating enzyme is a glycosylated protein. Although the ER contains many glycosylated proteins which also bind to ConA (Fig. 3.15), this column provides fair recovery of FGM activity and could be used for purification of FGly generating enzyme in combination with other columns.

3.4.6. Heparin Sepharose chromatography

Heparin is a straight-chain anionic glycosaminoglycan consisting of a repeating dimer of mostly alpha-L-iduuronic acid 2-sulfate, and 2-deoxy-2-sulfamino-alpha-D-glucopyranose 6-sulfate, with a molecular weight range of 5000-30000. It was shown that immobilized heparin can bind different proteins and thus, can be efficiently used as chromatographic media (Hata *et al*., 1993; Zhang *et al*., 1991; Moore *et al*., 1993; Bhikhabhai *et al*., 1992). Heparin Sepharose consists of highly cross-linked 6% agarose with coupled heparin molecules. Proteins are bound to immobilized heparin at low salt concentration and eluted with a gradient of high salt buffer.
For testing the suitability of heparin agarose for purification of FGly generating enzyme 120eq reticuloplasm diluted with OG buffer without KAc were mixed with Heparin Sepharose matrix and incubated for 30 min at 4°C. The flow through was collected and the gel was washed with OG buffer without KAc. Elution was performed by incubating of the gel with OG buffer containing 1M NaCl or 0,5M $(NH_4)_2SO_4$ for 30 min at RT. Flow through and eluate (Fig. 3.16) were tested for presence of FGM activity.
Absence of FGM activity in the flow through fraction showed that the enzyme was efficiently bound to immobilized heparin. The activity could be recovered in the salt eluates with 42% recovery in 1M NaCl eluate and 40% recovery in 0,5M $(NH_4)_2SO_4$ eluate.

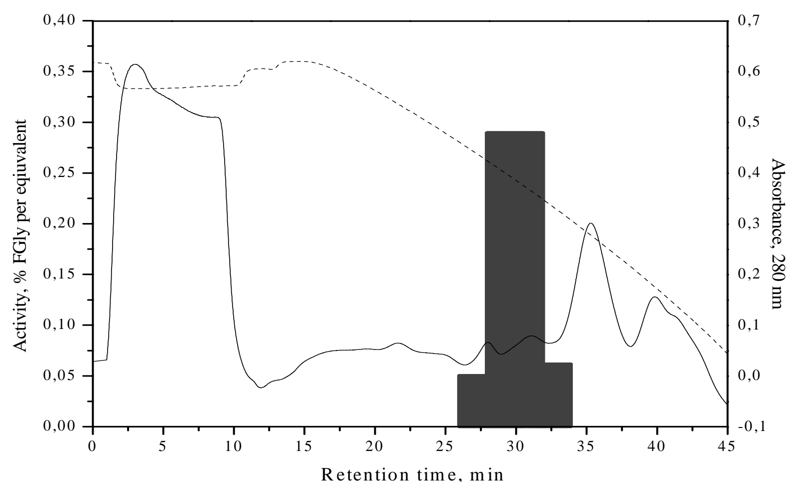
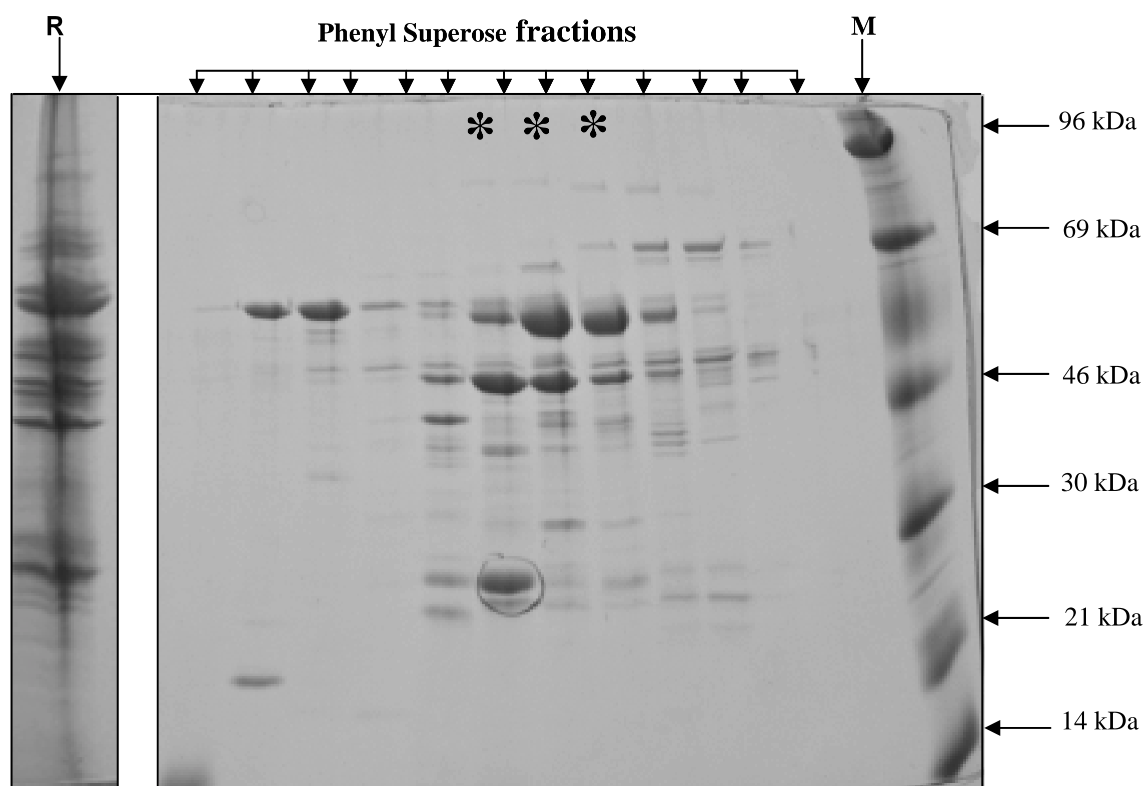


Fig. 3.11. Separation of reticuloplasm proteins on Phenyl Superose PC 1.6/5 hydrophobic interaction column at a starting salt concentration of 1.5 M $(NH_4)_2SO_4$. The dashed line shows the conductivity measured directly after passing the flow cell of the SMART system. Decreased starting concentration of salt led to weaker binding of FGly generating activity to the hydrophobic column matrix and to earlier elution.



*- fractions containing the FGly generating activity; M – molecular weight marker
R - reticuloplasm

Fig. 3.12. Separation of reticulolasm proteins on Phenyl Superose® PC 1.6/5 hydrophobic interaction column for Smart system (starting concentration of salt 1.5M). Proteins of 200µl reticuloplasm were separated on Phenyl Superose hydrophobic interaction column. Fractions of 100µl were collected and 15 µl of each fraction were applied on a 17,5% acrylamide SDS gel and stained with Coomassie blue.

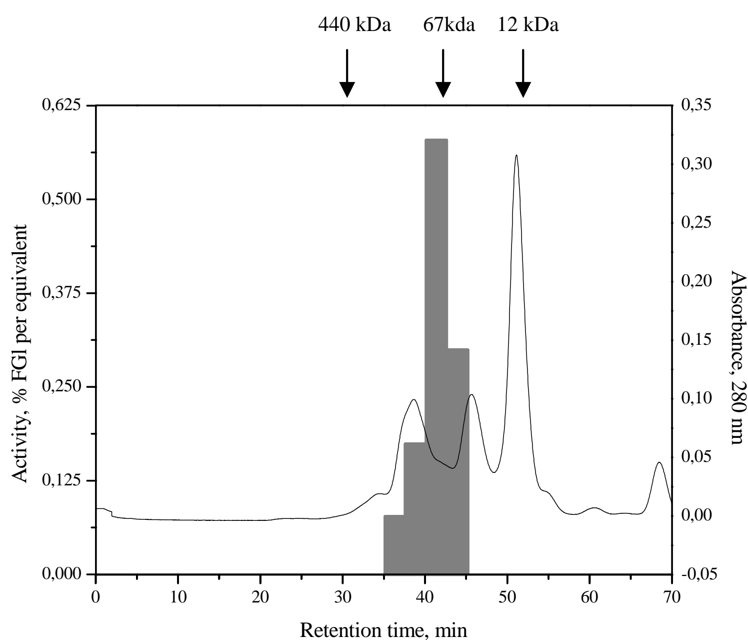


Fig.3.14. Separation of reticuloplasm proteins on Superdex 200 gel filtration column for Smart system. 50µl reticuloplasm were applied on Superdex 200 gel filtration column (column volume 2.4 ml) and eluted with OG buffer at a flow rate of 40µl per min. Activity elution profile is shown in grey bars. Arrows show elution profile of molecular weight standard proteins.

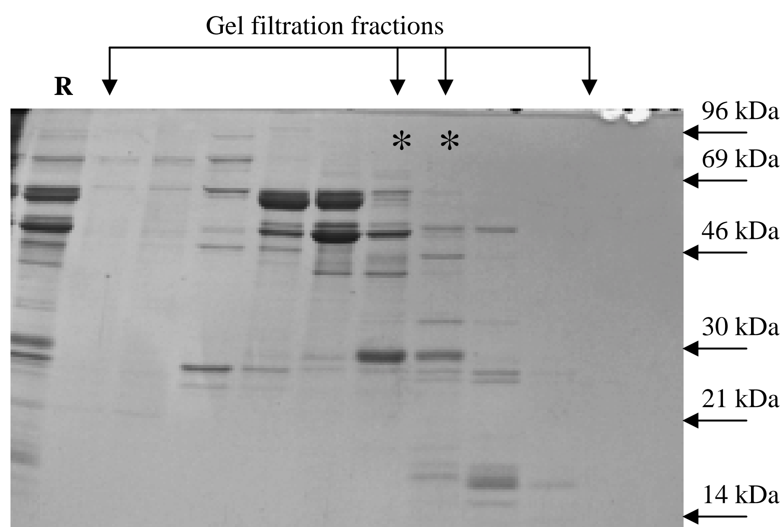
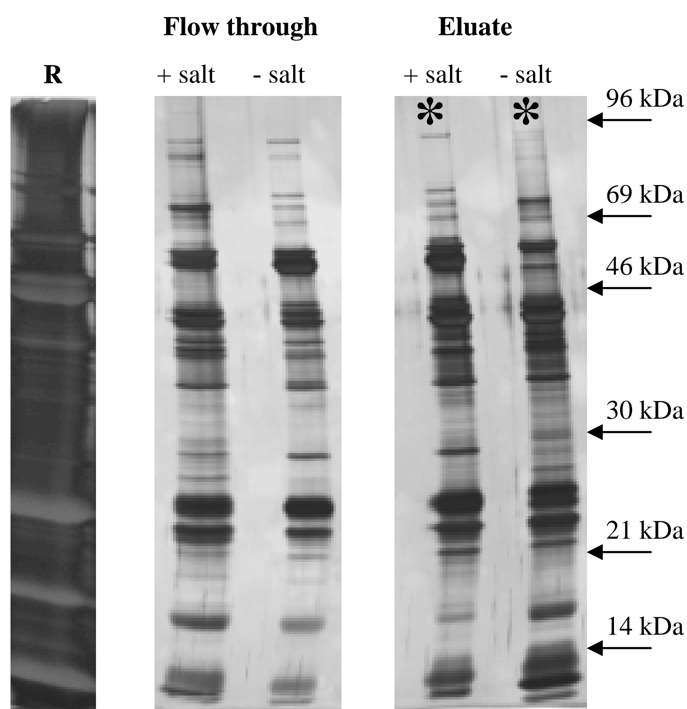


Fig. 3.14. Separation of the reticulolasm proteins on Superdex 200 gel filtration column for Smart system. Proteins of 50µl reticuloplasm were separated on Superdex 200 gel filtration column. Fractions of 100µl were collected and 15 µl of each fraction was analysed by SDS-PAGE (17,5% acrylamide). Separated proteins were visualised with Coomassie blue staining.



R – reticuloplasm
* - fractions, containing FGly generating activity

Fig.3.15. Separation of reticuloplasm proteins on Concavalin A Sepharose. Reticuloplasm was incubated with ConA Sepharose under different conditions (with and without salt, see text). Flow through and eluates were tested for FGly generating activity. Aliqiotes of reticuloplasm (60eq), flow through and eluates (corresponding to 20eq each) were analysed by SDS-PAGE (12,5% acrylamide). Separated proteins were visualised by silver staining. Protein profile of the flow through and the eluates from the ConA Sepharose show that many glycoproteins bind to the column matrix only partially. FGM, though, was bound quantitatively.

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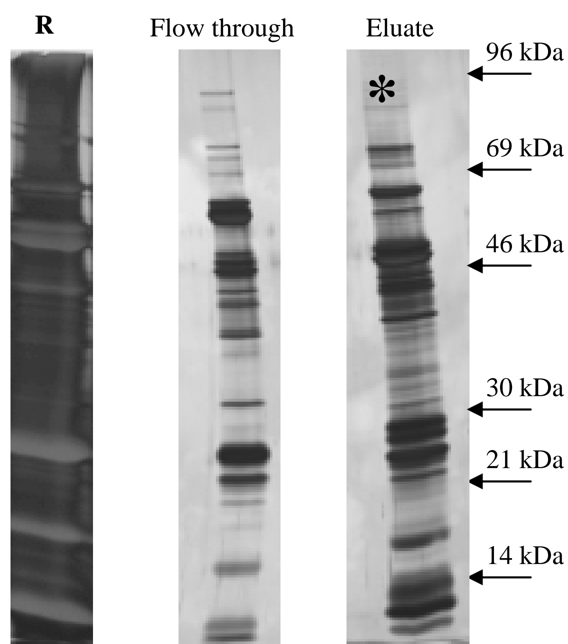


R – reticuloplasm
* - fraction, containing FGly generating activity

Fig. 3.16. Separation of reticuloplasm proteins on Heparin agarose. Reticuloplasm was bound to and eluted from Heparin agarose as described in the text. Flow through and eluates were tested for FGly generating activity. Aliquotes of reticuloplasm (60eq), flow through and 1M NaCl eluate (corresponding to 20eq each) were analysed by SDS-PAGE (12,5% acrylamide). Separated proteins were visualised by silver staining

3.4.7. Dye affinity matrix chromatography

Dye affinity matrices consist of agarose beads with coupled synthetic dyes, which are mimicking cofactors and natural substrates of the enzymes. These matrices are widely used for purification of proteins.

To test binding of FGM activity to the immobilized synthetic dyes 300eq reticuloplasm was subjected to buffer exchange to OG buffer without KAc, mixed with 100µl affinity matrices equilibrated with OG buffer and incubated for 30 min at 4°C with rotation. The flow through was collected and bound proteins were eluted from the gels with OG buffer containing 1M NaCl. The flow through and the eluate from each affinity matrix (Fig.3.17) were tested for FGM activity (see Table 3.4.7).

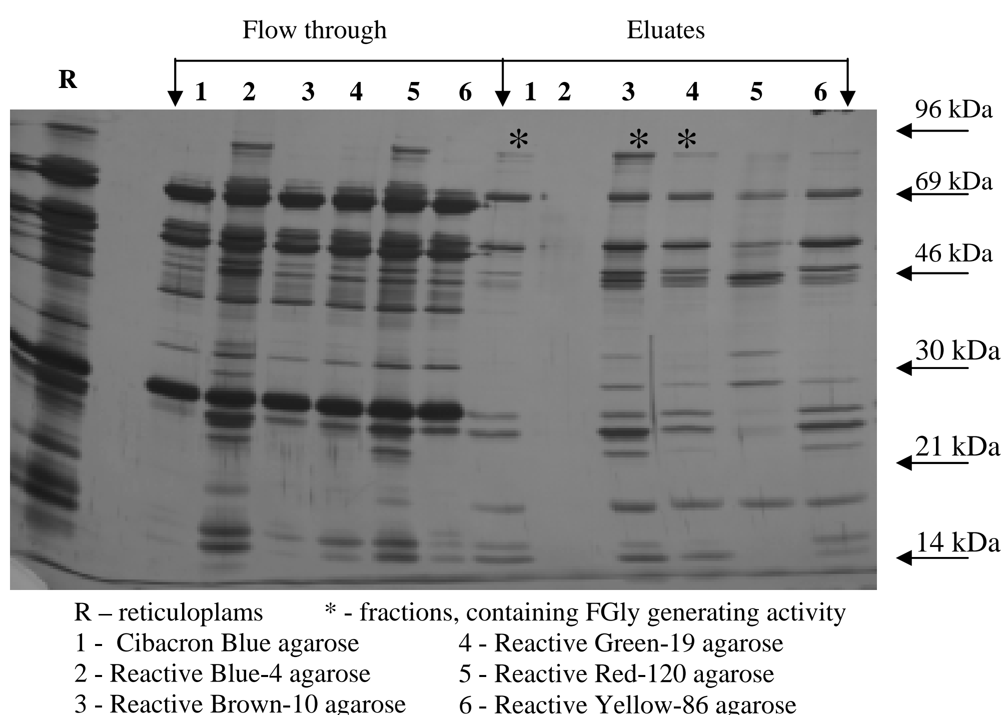


Fig.3.17. Separation of reticuloplasm proteins on the various affinity dye matrices. Equal aliquots of reticuloplasm, flow throughs and eluates from indicated dye affinity matrices were analysed by SDS-PAGE (12,5% acrylamide). For visualisation of proteins the gel was stained with silver.

Table 3.4.7. Recovery of FGM activity after chromatography on various dye affinity matrices

Dye Affinity matrix	FGM activity in the flow through	FGM activity in the eluate	Recovery of the bound FGM activity
Cibacron Blue agarose	-	+	13%
Reactive Blue-4 agarose	+	-	-
Reactive Brown-10 agarose	-	+	12%

Reactive Green-19 agarose	-	+	6%
Reactive Red-120 agarose	-	-	-
Reactive Yellow-86 agarose	+	-	-

3.4.8. Purification of FGM activity on affinity matrices with immobilised peptides

Affinity chromatography is one of the most efficient ways to purify proteins. As it was shown (see 3.3.6), the activity of FGM could be efficiently inhibited by low concentrations of ASA 65-80 native and ASA 65-80 C69S mutant peptides. It is supposed that FGM would bind to those peptides immobilised on the affinity support.

3.4.8.1. Binding of FGM activity to immobilized ASA peptides

Peptides ASA 65-80, ASA 65-80 C69S and ASA 65-80 „scrambled“ were immobilized on the AffiGel 10 affinity support at the concentration 5mg peptide per 1 ml of the gel. The ASA 65-80 „scrambled“ peptide was used as a control for the specificity of binding. 100 µl of the gel matrix with immobilized peptides was equilibrated with 5 volumes of OG buffer and then was incubated with 1500 eq reticuloplasm for 2 hours at 4°C with rotation. The flow through was collected and tested for presence of FGM activity (see Table 3.4.7.1).

Table 3.4.8.1. Recovery of FGM activity after immobilised peptides affinity chromatography

Peptide	Inhibition of FGM activity in the *in vitro* assay (see 3.3.6)	FGM activity in the flow through of immobilized peptide column
ASA 65-80	+	- (0% recovery)
ASA 65-80 C69S	+	- (0% recovery)
ASA 65-80 „scrambled“	-	+ (100% recovery)

FGM activity could not be detected in the flow through of the columns with immobilised peptides ASA 65-80 (later in the text C69) and ASA 65-80 C69S (later in the text C69S), though the flow through of the column with the „scrambled“ peptide (later in the text “scrambled affinity column”) showed no depletion of FGM activity. This corresponds well with the data of the peptide inhibition experiment (3.3.6). We concluded that FGly generating

enzyme was efficiently bound to the peptides presented on the affinity support and that binding was specific.

3.4.8.2. FGM activity can be eluted from C69S but not from the C69 affinity matrix

Absence of FGM activity in the flow through of the C69 and C69S affinity matrices could indicate that the FGly generating enzyme was bound to the immobilized peptides. We tried different elution methods to elute bound enzyme from both columns. As could be seen from Table 3.4.7.2, no elution method led to an active eluate in case of C69 affinity matrix. On the contrary, we could elute the enzyme in active state from the C69S affinity column using high concentrations of chaotropic salt in combination with high pH (2-4M KSCN, 50mM Tris pH 10.0) and also with 25µM ASA 65-80 C69S peptide. Both elution methods led to 20-40% recovery of FGM activity. Since high concentration of KSCN had an inhibitory effect on FGly formation, the high salt eluate had to be desalted through PD10 desalting column prior to measuring the activity of eluted enzyme. As desalting on PD10 column led to high degree of sample dilution we also tried dialysis in ultrathimbles UN 100/25 (Schleicher&Schuell), which allow concentration of the sample under vacuum with simultaneous dialysis. The recovery of activity was the same in both cases. The peptide eluate had to be well dialysed before measuring the FGM activity to remove the peptide. Concentration and dialysis was performed in ultrathimbles UN 100/25 (Schleicher&Schuell).

Table 3.4.8.2. Elution strategies

Elution Principle	**Elution Buffer**	**Activity of the Eluate (FGly, % of load)**	
		ASA 65-80	**ASA 65-80 C69S**
Low pH	Glycine 200 mM pH 2.2	**0.5**	**n.d.***
	Glycine 200 mM pH 2.8	**0.3**	**n.d.**
	Glycine 200 mM pH 3.5	**0.0**	**n.d.**
High pH	Tris 50 mM pH 9.0	**1.5**	**0**
	Tris 50 mM pH 9.75	**1.0**	**0**
	Tris 50 mM pH 10.5	**1.0**	**0**
	Tris 50 mM pH 11.5	**1.0**	**0**
	Tris 1 M pH 9.75	**0.0**	**0**
	Diethylamine 50 mM pH 10.5	**0.0**	**0**
Substrate Elution	25µM ASA 65-80 or ASA 65-80 C69S (subsequent dialysis)	**0.0**	**20-40**
High Salt	2.5M $MgCl_2$ pH 4.5	**0.0**	**n.d.**

	2M NaCl pH 8.0	**0.0**	**0.0**
	2M KSCN pH 9.75	**1.5**	**20-40**
	4M KSCN pH 9.75	**1.0**	**20-40**

* n.d. - not determined

The eluate from affinity matrix shows complex composition on SDS-PAGE, indicating that many proteins bind to immobilized peptides unspecificly (not shown). Although the eluate from affinity matrix provided good recovery of FGM activity, the complexity of its protein composition did not allow to use this column as a single purification step.

3.4.9. Activity and stability losses after partial purification of FGM

Though partial purification of FGM activity on the single chromatographic columns in some cases provided good activity recovery, in particular when activity was measured right after purification, these results turned out not to be reproducible. Activity recovery in the MonoQ-purified fractions, for example, could vary from 10% to 90%. We also discovered that stability of FGM activity in the purified fractions was severely decreased as compared to the starting material. When kept at room temperature for 4 hours, activity in MonoQ purified fractions dropped by 90%, though activity of reticuloplasm kept at the same conditions did not change (Fig.3.18).

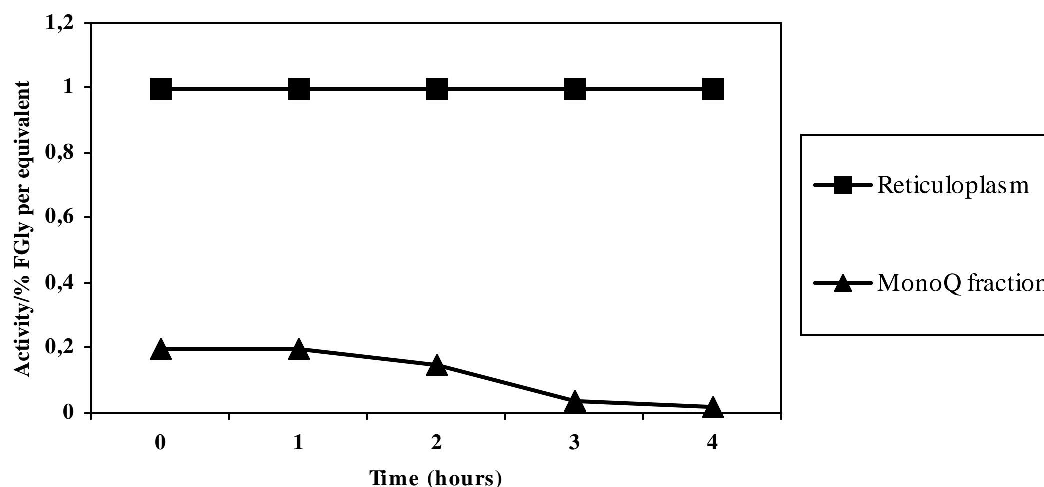


Fig. 3.18. Stability of FGM activity in reticuloplasm vs. MonoQ purifed fraction. Reticuloplam and MonoQ purified fraction were kept at room temperature for 4 hours. At the end of each hour an aliquot corresponding to 30eq reticuloplasm was withdrawn and tested for FGM activity. Recovery of FGM activity in MonoQ purified fraction was 20% of that of reticuloplasm when measured right after the run. Activity of reticuloplasm (indicated by squares) did not change, whereas activity in the MonoQ purified fraction (indicated by triangles) dropped almost 90% after 4 hours.

If kept at 4°C for 10-12 hours, MonoQ purified FGM completely lost FGly generating activity. Combination of two or three chromatographic steps led to 80-100% activity loss. For example, rechromatography of the MonoQ purified fractions on the same column led to practically total loss of activity (not shown). These facts might mean that FGly generating machinery required a component, presumably a cofactor, which was removed during different chromatographic steps, leading to irreversible activity loss. Low protein concentrations in the purified fractions and high dilution after some chromatographic steps could lead to unspecific losses of protein due to protein denaturation or adsorption to surfaces.

Purification on the affinity matrices with the immobilised peptides showed that FGly generating enzyme belongs to the minor component of the cell, since there was no obvious depletion of the major bands in the flow through after binding of FGM activity to the affinity matrix. Thus, in order to be able to purify the enzyme in amounts sufficient for identification one should start with a considerable amount of pancreas and perform purification on a big scale. However, purification on a big scale is lengthy and often requires intermediate freezing of the partly purified samples in addition to long dialysis between some chromatographic steps. Since this is only possible when activity of the purified components can "survive" all these treatments, we had to undertake a detailed investigation of stability of FGly generating machinery under different conditions and its sensitivity to cofactors and other low molecular weight components which could be present in the ER lumen. Another problem to solve was to improve activity (and protein) recoveries after different purification steps.

3.5. Optimisation of chromatographic steps for purification of FGM activity

The results below were obtained using anion exchanger purified fraction containing FGM activity after separation of reticuloplasm on the MonoQ® PC 1.6/5 and MonoQ® HR 5/5 columns and eluate from C69S affinity matrix.

3.5.1. FGM activity in purified fractions is improved by a heat stable factor present in reticuloplasm

One of the possible ways to find out whether reticuloplasm contained a low molecular weight component that is important for FGM activity was to separate reticuloplasm proteins from low molecular weight components by gel filtration. 120 eq reticuloplasm were boiled for 5 min at 95°C and passed over a Fast Desalting PC 3.2/10 gel filtration column in OG buffer,

the protein peak and "salt" peak (containing low molecular weight components) were collected. Boiling leads to denaturation of the proteins but is not harmful for most low molecular weight compounds. A part (10%) of the protein peak and the low molecular weight components peak from both runs were added to aliquots of the MonoQ PC 1.6/5 purified fraction (after separation of 300eq reticuloplasm) during incubation with RNCs and puromycin under standard assay conditions. As a control the MonoQ purified fraction was incubated with 15µl reticuloplasm boiled at 95°C. We observed that boiled reticuloplasm dramatically simulated activity in the MonoQ fraction. Addition of the gel filtration separated protein and low molecular weight components of reticuloplasm showed that an activating component was associated with the denatured protein peak (Fig.3.19). Substitution of boiled reticuloplasm by boiled BSA or reticulocyte lysate (25µg added during incubation with RNCs) did not improve the activity of MonoQ purified fraction.

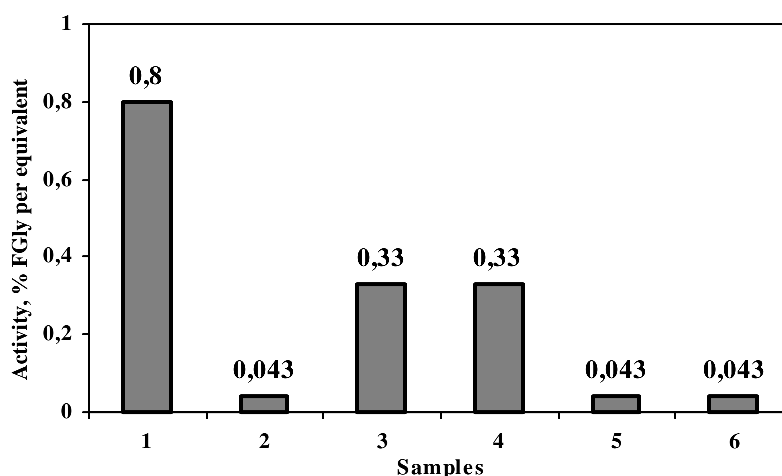


Fig. 3.19. Stimulation of FGM activity in MonoQ purified fraction by a heat-stable factor present in the reticuloplasm. Reticuloplasm (with activity 0.8% FGly per eq, 1) was subjected to boiling at 95°C, which led to inactivation. MonoQ purified fraction (2, activity 0,043% FGly per eq, which corresponds to 6% recovery as compared to reticuloplasm) was incubated with boiled reticuloplasm (3), protein peak of gel filtration separated boiled reticuloplasm (4), 25µg of boiled BSA (5) and reticulocyte lysate (6). Only boiled reticuloplasm and the protein peak after gel filtration of boiled reticuloplasm stimulated activity in purified MonoQ fraction up to 0,33 % FGly per equivalent (7,7 fold stimulation).

As boiling led to total inactivation of reticuloplasm (in terms of FGM activity), it is not likely that the activation effect was associated with a protein function. But it could well be a cofactor or other low molecular weight compound stably associated with the proteins but not destroyed by boiling. Thus, the FGly activity enhancing effect was associated with ER proteins (or with protein associated cofactors) and showed high heat resistance.

3.5.2. Effect of cofactors and calcium on FGM activity in purified MONOQ fractions

To investigate the influence of different cofactors, aliquots of MonoQ purified fraction were incubated with RNCs and puromycin in the presence of a cofactor mix or single cofactors (Table 3.5.2).

Table 3.5.2. Effect of different cofactors on FGM activity in MonoQ purified fraction

Cofactors	Concentration in the assay	Effect on MonoQ purified fraction
NAD	0.5 mM	no
NADP	0.5 mM	no
NADPH	0.5 mM	no
FAD	0.5 mM	no
FMN	0.5 mM	no
Biopterin	0.5 mM	no
Thiamin PP	0.5 mM	no
PQQ	0.5 mM	no
GSH	1.25-2.5 mM	stimulation
GSSG	1 mM	inhibition

The results showed that only GSH was stimulating FGM activity in the purified fraction, whereas GSSG had inhibitory effect (Fig.3.20). Further experiments showed that GSH stimulated FGM activity when present during the assay in concentrations up to 2.5 mM, higher concentrations (more than 5mM) had an inhibitory effect (Fig.3.21). Similar results were obtained by substituting GSH by DTT (Fig.3.21) Optimisation studies showed that the optimal concentration of both GSH or DTT during the assay was 2.5mM.

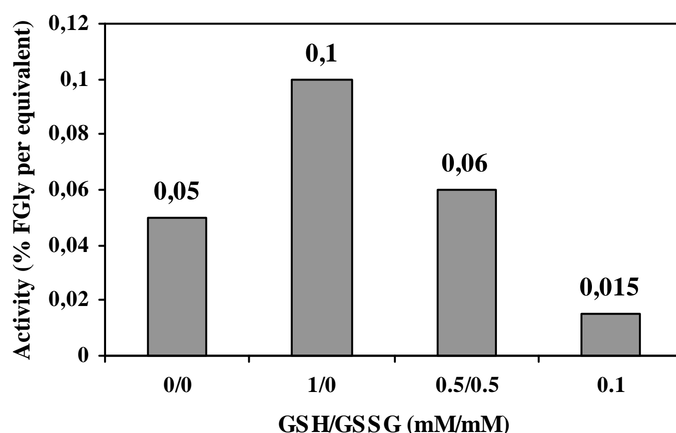


Fig. 3.20. Stimulation of FGM Activity in the MonoQ purified fraction by GSH/GSS
Equal aliquots of MonoQ purified fraction were assayed in the pre-sence of different conce of GSSG inhibited FGly generating activity, and 1 mM G

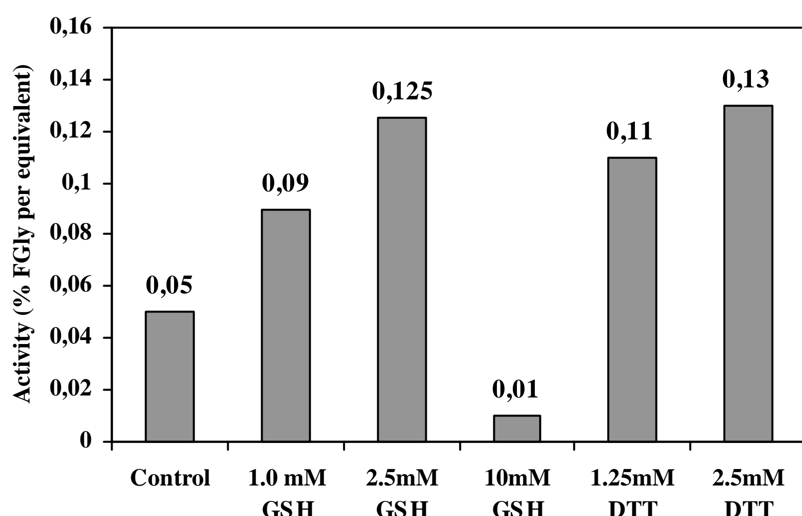


Fig. 3.21. Stimulation of FGM activity in MonoQ purified frac-tion by GSH and DTT. Equal aliquots of the MonoQ purified fraction were assayed for FGly generating activity under standard assay conditions in the presence of different concentrations of GSH or DTT.

To test whether thiols of GSH/DTT were required to prevent oxidation of enzyme cysteines or whether they had antioxidising effect on the cysteine to be modified in the *in vitro* ASA substrate 2.5mM DTT were added to all the buffers used for RNCs preparation (see Methods). The results (Fig. 3.22) showed that substrate prepared in the presence of DTT did not improve activity in MonoQ purified fraction and even had some inhibitory effect. Thus we concluded that DTT was required to maintain the active state of the purified FGly generating enzyme. Stimulation of activity by DTT could possibly explain stimulating effect of the boiled reticuloplasm: exposed SH-groups of the denatured proteins could perform the same effect as SH-groups of DTT, possibly preventing oxidation of the enzyme cysteines.

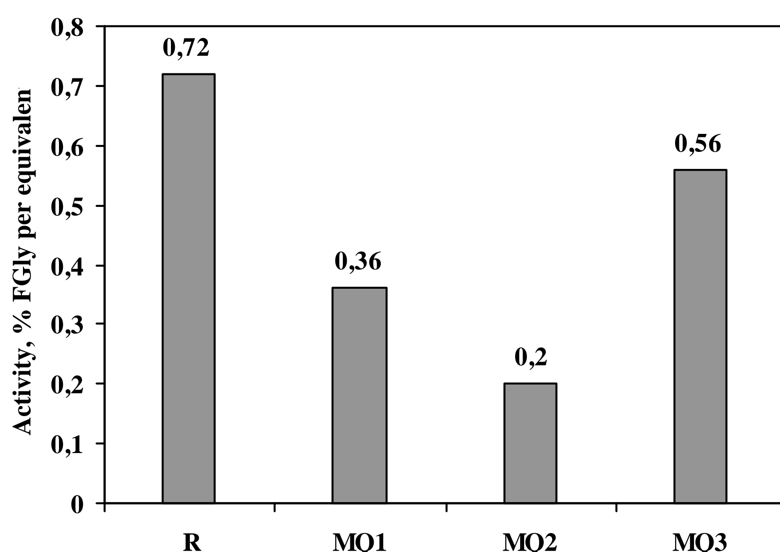


Fig. 3.22. DTT added during substrate preparation has no stimulatory effect.
Reticuloplasm proteins were separated on the MonoQ® PC 1.6/5 (0.1ml) column. FGM activity was measured with the RNC substrate prepared in the presence of 2.5 mM DTT (MQ2) and with the substrate prepared in the normal way (MQ1). DTT had a stimulatory effect on FGly generating activity in the purified fraction only when present during chromatography (MQ3). See also Fig. 3.22.

The ER has a much higher concentration of Ca^{2+} than cytosol and many of Ca^{2+} binding proteins are found in the ER lumen. To investigate the influence of Ca^{2+} on activity in the purified fractions the aliquots of the MonoQ purified FGM were incubated with RNCs and puromycin in presence of different concentrations (0-15µM) of $CaCl_2$. Fig. 3.23 shows that at a concentration of 15µM, Ca^{2+} ions stimulated the activity of the purified fraction almost 3-fold. Increasing concentration of Ca^{2+} ions further did not result in higher activating effect (not shown).

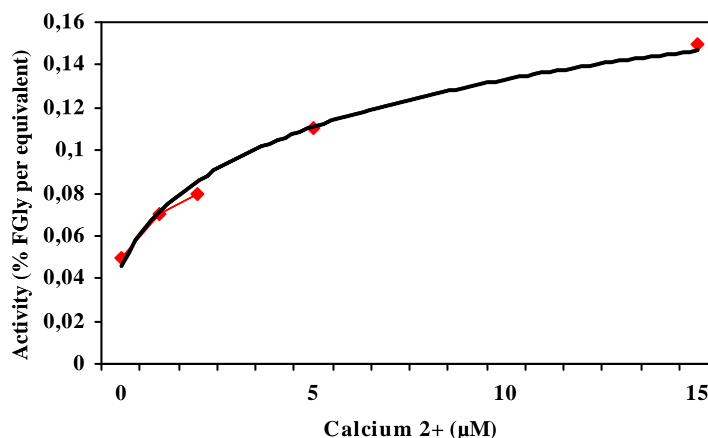


Fig. 3.23. Stimulation of FGM activity in the purified MonoQ fraction by calcium. Equal aliquots of MonoQ purified fraction were tested for FGM activity under standard assay conditions in the presence of different concentrations (0-15 µM) of $CaCl_2$, as indicated. 15 µM $CaCl_2$ stimulated activity almost 4-fold. At higher $CaCl_2$ concentrations (up to 100µM) similar modification efficiencies were observed as at 15 µM $CaCl_2$ (not shown).

3.5.3. DTT and Ca^{2+} ions are required during chromatographic purification for best recovery of FGM activity

The next question to answer was whether DTT and Ca^{2+} ions are required during purification to improve recovery of FGM activity or whether they fully "reactivated" FGM when added to the assay mixture. 300 eq reticuloplasm were separated on MonoQ® PC 1.6/5 using buffers containing 2.5mM DTT and 15µM $CaCl_2$. The FGM activity of the purified fraction was compared with that of MonoQ fraction obtained without addition of DTT and $CaCl_2$ to the buffers and with that of MonoQ fraction supplemented with DTT and $CaCl_2$ only during incubation with RNCs and puromycin. The results showed that DTT and $CaCl_2$ had the best stimulatory effect when added to the chromatographic buffers. Similar results were obtained for gel filtration chromatography (Fig.3.24). The purified fraction from hydrophobic interactions column did not show any difference in terms of activity recovery between the runs performed in the presence and in the absence of DTT/ $CaCl_2$.

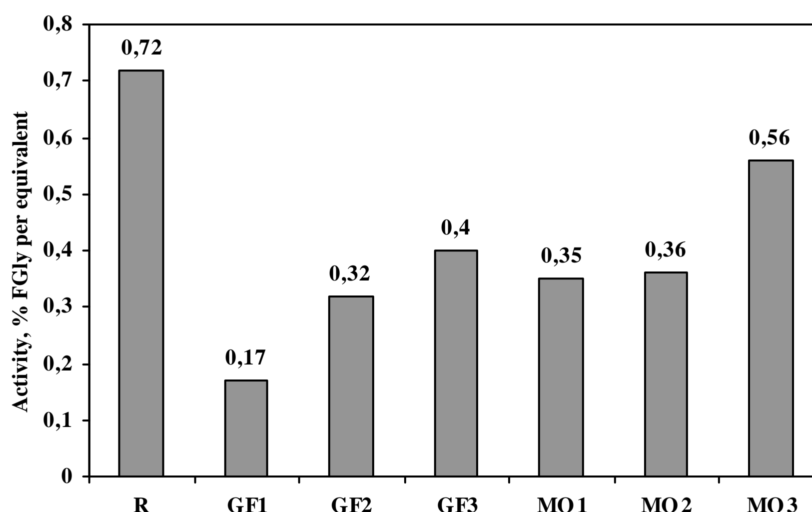


Fig. 3.24. Stimulatory effect of DTT and calcium when added to chromatography buffers. Reticuloplasm (R) proteins were separated on a Superdex 200 gel filtration column (GF) and MonoQ (MQ) anion exchanger column using the Smart system. Chromatography for samples 1 and 2 was performed without addition of DTT and calcium to the buffers, for samples 3 with the buffers containing 2.5 mM DTT and 15µM $CaCl_2$. For samples 2, DTT (final concentration 2.5 mM) and $CaCl_2$ (final concentration 15µM) were added during incubation with RNCs and puromycin. .

3.5.4. DTT increases stability of purified MonoQ fractions during storage and freezing

One of the problems encountered during combination of different chromatographic steps was the instability of FGM activity when the purified fractions were kept on ice, after freezing/thawing or when subjected to prolonged dialysis. We tested whether the stability of FGM activity would improve after addition of DTT to chromatographic buffers. Reticuloplasm and MonoQ purified fraction (2.5 mM DTT added to chromatographic buffers) were kept on ice for period of 7 to 10 days, the aliquots for incubation with RNCs and puromycin were taken each day. Analysis of the FGM activity of reticuloplasm and purified fraction after keeping on ice showed that addition of DTT dramatically increased stability of the purified fraction (Fig.3.25). The activity of FGM in purified fraction remained unchanged when kept on ice for up to 24 hours and decreased by 50% after 4 days.

Resistance of the MonoQ purified fraction to freezing-thawing was tested by measuring the FGM activity in the aliquots of MonoQ fraction after 3 cycles of shock freezing in liquid nitrogen. Each time the fraction was thawed on ice and then frozen again. Analysis of FGM activity showed that MonoQ purified enzyme could survive three cycles of deep freezing without activity loss.

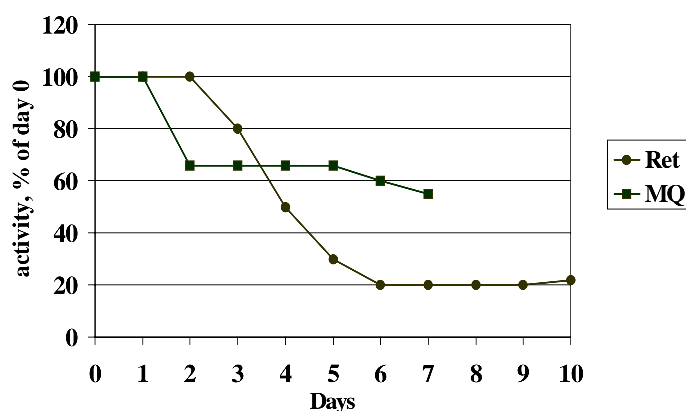


Fig. 3.25. Stability of reticuloplasm and MonoQ purified active fraction (2.5mM DTT present during chromatography) at 4°C. Reticuloplasm and MonoQ purified fraction containing FGM activity were kept on ice for a period of up to 10 days. Every day the aliquot was withdrawn and assayed for FGM activity. The activity in reticuloplasm was stable for 2 days and decreased by 50% after 4 days on ice. Activity in MonoQ purified fraction was stable for 24 hours, but decreased by 50% after 7 days keeping on ice.

3.5.5. Improvement of recovery of two-step purified enzyme by addition of BSA, cytochrom c and amino acid mix

Combination of anion exchanger and C69S affinity column has proved to be most efficient in terms of purity of the outcoming active fraction. However, we observed almost 100% loss of activity which may be due to unspecific protein loss, since after the second purification step (affinity column) the protein concentration in the purified fraction was extremely low,.

Unspecific loss due to interactions of proteins with surrounding surfaces is one of the problems one has to deal with when purifying low abundant proteins. One of the ways to solve this problem is to add a carrier protein to the solutions, which will cover the surfaces, thereby preventing binding (loss) of protein of interest. Another opportunity is to cover the surfaces with a solution of a detergent (for example, low concentration of Tween 20) or carrier protein.

We tested whether recovery of protein/activity could be improved by addition of carrier proteins or other components to the solutions used in the purification procedure.

7500 µl reticuloplasm was passed over MonoQ HR 5/5 anion exchange column, using buffers containing 2.5mM DTT. The fractions containing FGM activity were applied on the C69S affinity column and incubated for 2.5 hours at 4°C. After collecting the flow through and washing of the affinity matrix bound proteins were eluted with elution buffer containing 25µM ASA 65-80 C69S peptide (2x2 column volumes, 30 min each elution). The eluate was split into 6 equal parts and each part concentrated in a separate ultrathimble UN 100/25

(Schleicher&Schuell) to 200µl and then dialysed overnight against elution buffer without peptide. Ultrathimbles were treated in different ways to avoid unspecific loss of protein. The resulting dialysed eluate was tested for FGM activity (for recoveries see Table.3.5.5).

Table 3.5.5. Recovery of MonoQ/affinity purified FGM activity after concentration in ultrathimbles

Treatment of the ultrathimble	Recovery of FGM activity (% of activity loaded to MonoQ)
No treatment	0.3
0.5 ml of 0.1mg/ml BSA added to ultrathimble 30 min before sample and present during concentration and dialysis	25
0.5 ml of 0.1 mg/ml BSA added to ultra thimble 30 min before sample, removed before concentration and dialysis ("BSA-coated ultra thimble")	6
0.5 ml of 0.1mg/ml Cytochrome c added to ultra thimble 30 min before sample and present during concentration and dialysis	25
0.5 ml 0.02 % Tween added to ultra thimble 30 min before sample, 0.02 % Tween present in the dialysis buffer	3
0.5 ml 1mg/ml amino acids* mix added to ultra thimble 30 min before sample, 1mg/ml amino acids* mix present in the dialysis buffer	20

*Mix of 20 amino acids in equal proportion with a final concentration of 1mg/ml

All additions or pre-treatment of ultra thimbles significantly improved recovery of FGM activity. That proves that activity loss was actually associated mainly with protein loss and not with protein inactivation. The best recovery was obtained by addition of carrier proteins or amino acid mix to the sample during dialysis. For purification purposes the latter was the best, as amino acids, unlike carrier proteins, introduce no protein contaminations to the

purified enzyme and would not interfere with subsequent protein identification. For best protein recovery and to avoid possible losses in later experiments the amino acids mix with a concentration of 1mg/ml was included in the elution buffer already during elution of FGM activity from the affinity matrix.

3.6. Strategies for purification of FGly generating enzyme

Having optimised the single chromatographic steps in terms of activity and protein recovery we tried to develop a strategy for purification of FGM activity on a big scale. Since FGly generating enzyme is apparently a low abundant protein of the ER, starting with high amounts of pancreas was necessary to get a signal on SDS PAGE which would be sufficient for the following identification of the purified proteins by mass spectrometry. One should also choose the most efficient (in terms of purification factor) and fastest way of purification to reduce losses of activity due to thermal inactivation.

Having compared the purification factors of different chromatographic columns for FGM activity we have chosen the MonoQ column as one preparative step. MonoQ provided good and precise separation of reticuloplasm proteins and could be used in HR 10/10 format for purification of FGM activity on a big scale (up to 40 ml reticuloplasm in one run, corresponding to approximately 120 mg total protein). MonoQ provided good recovery of activity (40-80% recovery) and a high purification factor (see 3.4.1). Another column providing good recoveries of activity and a very high purification factor was the affinity matrix with immobilised ASA 65-80 C69S peptide. The affinity matrix with immobilised ASA 65-80 "scrambled" peptide could be used as a kind of "precolumn" allowing to "pick up" the unspecificly binding proteins, thereby decreasing the background in the specific affinity column eluate.

Several strategies were developed for purification of FGM activity on a big scale involving the mentioned above chromatographic columns.

3.6.1. FGM purification by combination of MonoQ® PC 1.6/5 anion exchange column and C69S affinity column and identification of purified proteins

Microsomes prepared from 50g of bovine pancreas were subjected to detergent treatment to extract lumenal components of the ER with the outcome of 42 ml reticuloplasm (120 mg total protein). Reticuloplasm proteins were separated on the MonoQ® PC 1.6/5 column with the buffers containing 2.5 mM DTT in 40 runs, 1 ml reticuloplasm in each run. Fractions

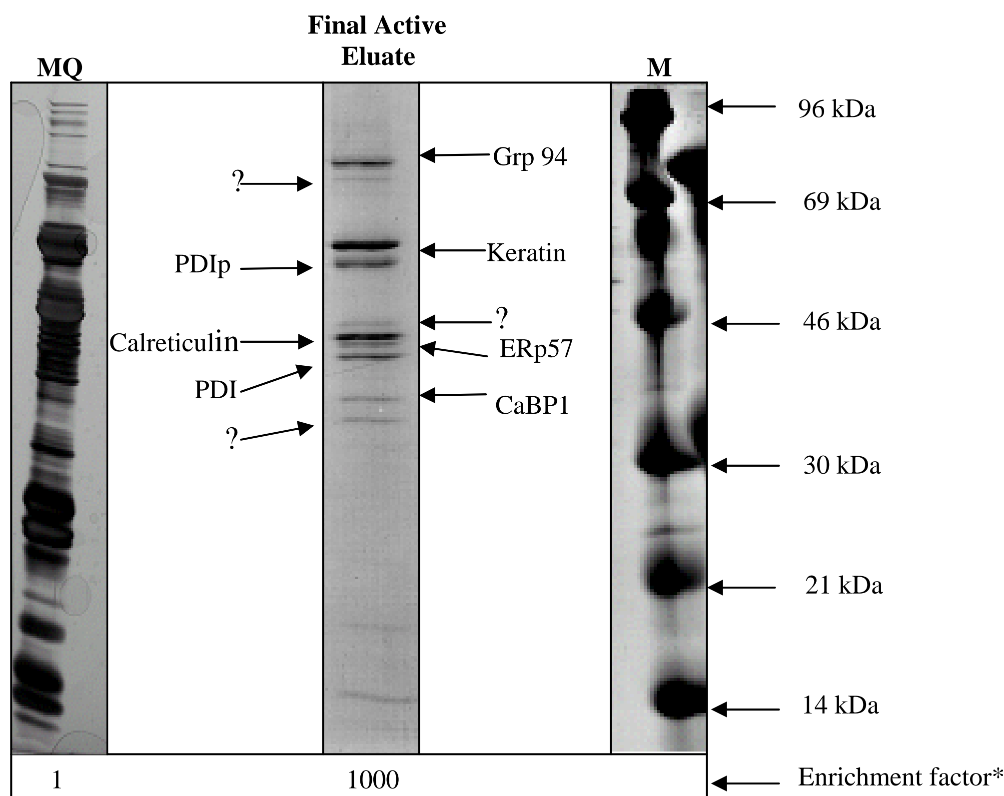
containing FGM activity (see 3.4.1.2) were shock frozen in liquid nitrogen directly after the run and kept at –80°C.

The frozen fractions were thawed on ice and pooled (107 ml pool). This MonoQ pool was applied on 4.5 ml C69S affinity matrix equilibrated with incubation buffer (20 mM Tris pH 8.0, 200 mM NaCl, 2.5 mM DTT) and incubated for 3 hours at 4°C with rotation. After collecting the flow through the column was washed with incubation buffer containing 2M NaCl to remove unbound and weakly bound proteins. The column was eluted with 6 column volumes (2 times 3 column volumes for 30 min) of elution buffer (2M KSCN, 50mM Tris pH 10.0). The eluate was desalted on a PD10 gel filtration column and, after desalting, applied again on 1.5 ml C69S affinity column and incubated for 3 hours at 4°C with rotation.

The rechromatography of the eluate on the same affinity matrix was performed to remove unspecificly bound proteins and also to concentrate the final eluate containing FGM activity. The eluate from the second affinity column was desalted again on a PD10 gel filtration column, concentrated to 25µl in a Vivaspin concentrator and boiled directly in the concentrator with 25µl 2 fold Laemmli buffer before being analysed by SDS PAGE. The gel was stained with Coomassie Blue overnight. The visualised bands were extracted from the gel, subjected to in-gel-digestion with trypsin and analysed by MALDI-TOF mass spectrometry (Fig. 3.26).

Table. 3.6.1. Recovery of FGM activity during purification

Purification steps	Activity (% FGly modification per equivalent)	Recovery of FGM activity (% of loaded activity)	Recovery of FGM activity (% of reticuloplasm activity)
Reticuloplasm	1.1	100	100
MonoQ purified pool	0.53	48	48
First Affinity column flow through	0	0	0
First affinity column eluate	0.12	23	10
Second affinity column flow through	0	0	0
Second affinity column eluate	0.1	83	9



MQ – MonoQ purified fraction
M – molecurar weight marker
PDIp – pancreas specific protein disulphide isomerase
PDI – protein disulfate isomerase
CaBP1 – calcium binding protein 1
Grp94 – glucose regulated protein 94

* - a 1000 fold larger aliquot of the final eluate was loaded on the gel as compared to the MonoQ eluate

Fig. 3.26. Purification of FGM activity by combination of MonoQ PC 1.6/5 anion exchange column and C69S affinity column. FGM activity from 50g bovine pancreas was purified over MonoQ anion exchanger column and peptide affinity column (see text). The purified fraction was applied on a 12,5% acrylamide SDS-PAG and separated proteins were visualised by Coomassie blue staining. Identified bands are indicated by arrows. Some bands (indicated by question mark) could not be identified due to insufficient amount of protein or incompleteness of databases for bovine proteins.

As shown in the Table 3.6.1, FGM activity was efficiently bound to both affinity columns (activity was completely absent from the flow through). The final outcome of activity in the last eluate was only 9% of that of the starting material. Identification of the purified proteins revealed one strong keratin band, four proteins (PDIp, ERp57, PDI and CaBP1) belonging to the protein disulphide isomerase (PDI) family, GRP 94 and calreticulin. Identity of other bands could not be determined either due to insufficient amount of protein on the gel or due to incompleteness of the databases for bovine proteins. Interestingly, all identified proteins (except contaminating keratin) perform chaperon functions in the ER and have high affinity to peptides. Although a possible role of chaperones in FGly modification can not be excluded,

these proteins may also bind to the peptides presented on the affinity matrix unspecificly. That is why in the next two purification approaches we introduced the peptide column with scrambled ASA peptide, to reduce the background of unspecificly binding proteins. In the next approaches we also tried to improve recovery of FGM activity in the affinity eluates by introducing the amino acid mix (see 3.5.5) to the elution and dialysis buffers.

3.6.2. FGM purification by combination of MonoQ® HR 10/10, "scrambled" peptide column and C69S affinity columns, and identification of purified proteins

3.6.2.1. Approach 1 („+ and - approach")

Microsomes prepared from 300 g of bovine pancreas were subjected to detergent treatment to extract lumenal components of the ER with the outcome of 260 ml reticuloplasm (780 000 eq, 800 mg total protein). Reticuloplasm proteins were separated on the MonoQ® HR 10/10 column with the buffers containing 2.5 mM DTT. In a single run 30 ml reticuloplasm (45 000 eq) were applied on the column. Fractions containing FGM activity (see 3.4.1.2) were shock frozen in liquid nitrogen directly after the run and kept at –80°C.

The frozen fractions were thawed on ice and pooled (32.5ml pool). To reduce the volume, the MonoQ fractions pool was concentrated to 10.5 ml using Vivaspin concentrators at room temperature. The concentrated MonoQ pool was applied on a 6 ml "scrambled" peptide column equilibrated with the incubation buffer (20mM Tris pH 8.0, 200mM NaCl, 2.5mM DTT) and incubated at 4°C overnight with rotation. The flow through of the scrambled peptide column was directly applied on 4 ml C69S affinity column equilibrated with the incubation buffer and incubated for 3 hours at 4°C with rotation. After collecting the flow through, the column was washed with incubation buffer and incubation buffer containing 2M NaCl to remove unbound and weakly bound proteins. The column was eluted with 6 column volumes (3 times 2 column volumes for 3 times 2 hours) of 25µM ASA 65-80C69S peptide in the incubation buffer containing 1mg/ml amino acid mix. The eluate (24 ml) was split in two equal parts (12 ml each). One part was concentrated in the Vivaspin concentrator to 25 µl, boiled with 25µl 2 fold Laemmli buffer directly in the concentrator and applied on a 15% acrylamide SDS PAG. The other part was concentrated in the ultrathimble UH 100/25 to 0.6 ml and dialysed overnight against incubation buffer containing 1mg/ml amino acid mix. During dialysis the peptide was removed from the eluate. Dialysed eluate was applied on a 0.6 ml C69S affinity matrix. The flow through of the second affinity column was concentrated

in Vivaspin concentrator and applied on 15% acrylamide SDS PAG. The column was eluted with 6 column volume incubation buffer containing 25µM C69S peptide.
The main idea of splitting the eluate of the affinity column was to have a kind of “+” and “-” lanes on the SDS gel. The “+” lane comprises the first part of the eluate, containing FGM activity. The “-” lane comprises the flow through of the second affinity column, that should be depleted of FGM activity. Since all the FGM activity of the first affinity column eluate was bound to the second affinity column (see Table 3.6.2.1), one should be able to compare the lanes directly and analyse the bands present in the “+” lane and absent in the “-” lane (Fig. 3.27).
After separation of the proteins by SDS PAGE the gel was stained overnight with Ruby Red Fluorescent dye to visualise the proteins (Fig.3.27). For subsequent extraction of the bands and identification by mass spectrometry, the gel was incubated for 2-3 days in Roti-Blue Colloidal Coomassie solution. Protein bands were cut out of the gel and subjected to in-gel tryptic digestion and identification by MALDI-TOF mass spectrometry.

Table. 3.6.2.1. Recovery of FGM activity during purification

Purification steps	**Activity (% FGly modification per equivalent)**	**Recovery of FGM activity (% of loaded activity)**	**Recovery of FGM activity (% of reticuloplasm activity)**
Reticuloplasm	2.15	100	100
MonoQ purified pool	0.9	41	41
Unspecific column flow through	0.7	77	33
First Affinity column flow through	0	0	0
First affinity column eluate	0.07	10	3.3
Second affinity column flow through	0	0	0

Table 3.6.2.1 shows that scrambled peptide column introduces some loss (about 8%) of FGM activity. Recovery of activity in the eluate of the first affinity column was very low (only 3.3% of activity of starting reticuloplasm). Low recovery could not be prevented by adding the amino acid mix to the elution and dialysis buffers. The activity loss may be associated with the high dilution factor and very low protein concentration in the eluate. It can be partly

explained also by thermal inactivation and protein losses due to the adsorption to the surfaces. These two factors could also be the reason for some activity loss (about 8%) in the scrambled peptide column flow through. To reduce the surface adsorption factor in the next approach we reduced the amount of both scrambled peptide column and C69S affinity column. We also tried to avoid losses of protein by elution of the proteins bound to affinity matrix directly with Laemmli buffer. This eluate could be directly analysed by SDS PAGE.

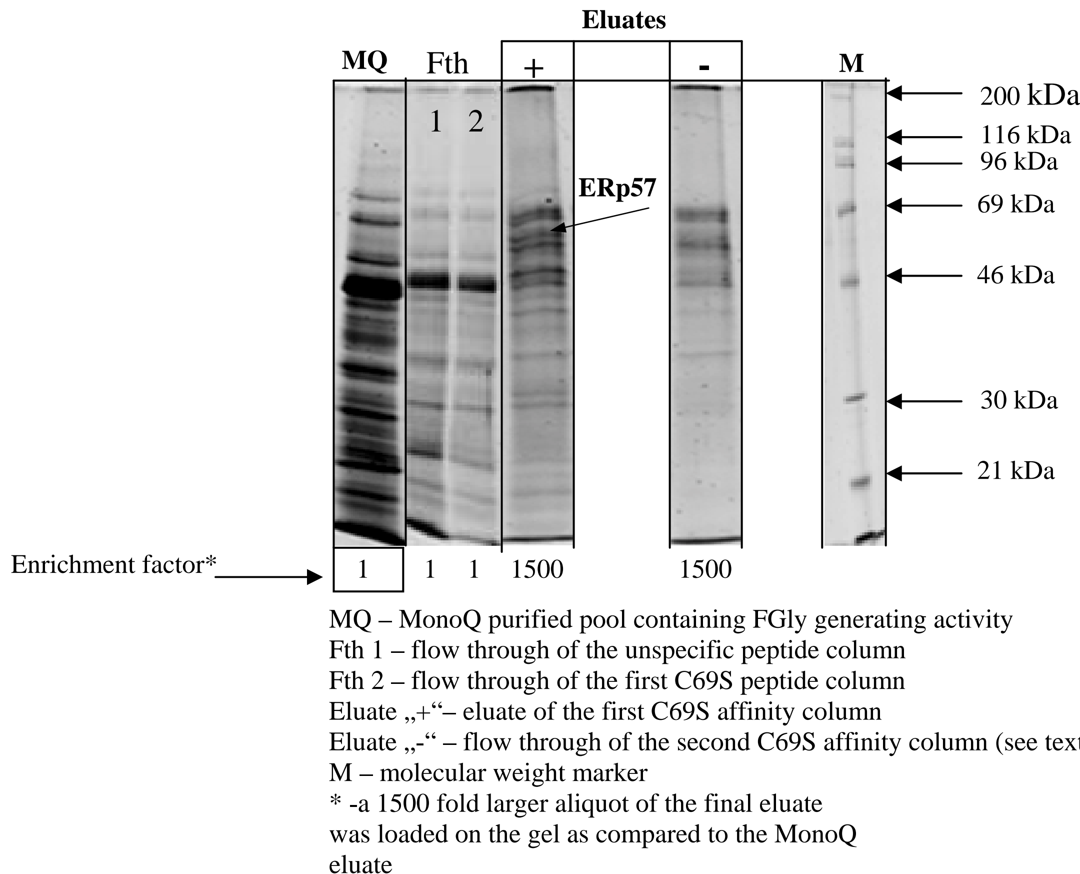


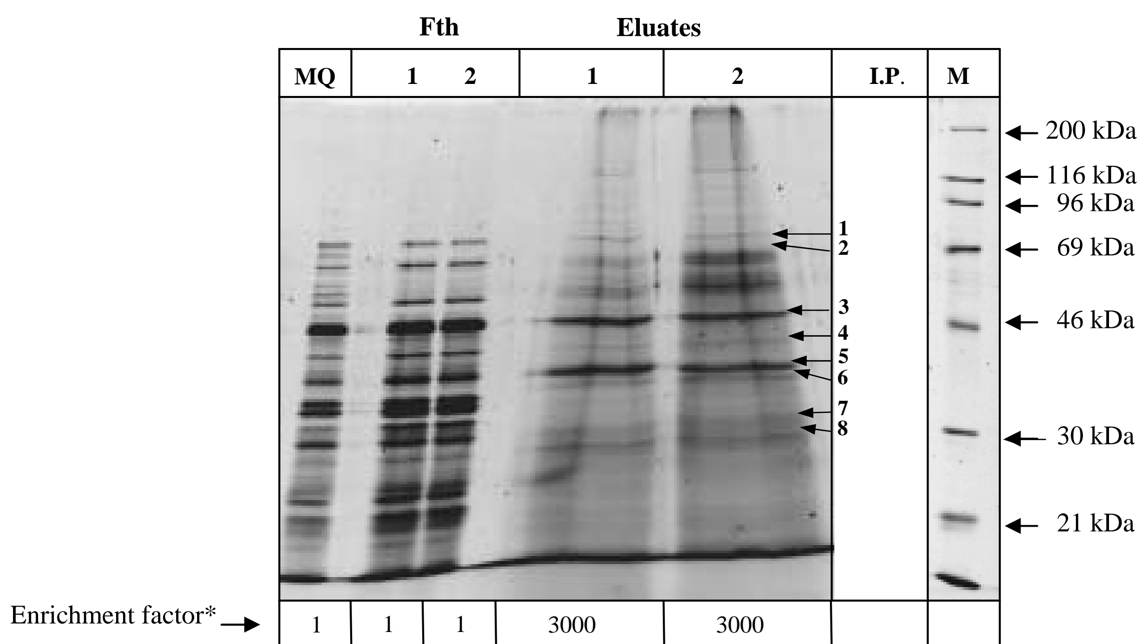
Fig. 3.27. Purification of FGM activity by „+ and - approach“.
FGM activity from 300g bovine pancreas was purified over MonoQ column, scrambled peptide column, and the C69S affinity column. The active eluate was split into two halves, one of which was concentrated directly for SDS-PAGE (eluate „+“) and the other one was dialysed and passed again over C69S affinity column for removal of FGM activity (eluate „-“, see also text). Proteins were applied on a 15% acrylamide SDS-PAG and visualised after separation by Ruby Red fluorescent dye. Comparison of the active eluate „+“and inactive eluate „-“ revealed some protein bands present on the former and absent in the latter one. Analysis by mass spectrometry led to identification of a PDI homolog in the active eluate, which was absent in the inactive one. Other protein bands were either identified as human keratin or could not be identified.

After comparison of protein composition of the “+” and “-” eluates we tried to identify the bands present in the former and absent in the latter one. We could determine the identity of only of one band, which turned out to be ERp57, a protein belonging to the PDI protein family. The identity of other bands could not be identified due to very strong keratin

contamination of the gel. High background created by keratin tryptic peptides masked cryptic peptides of other proteins, making their identification impossible. The direct elution of proteins bound to the affinity matrix with SDS-containing Laemmli buffer in the next approach could increase amount of protein of interest on the SDS gel.

3.6.2.2. Approach 2 (elution with SDS as the last step)

The first steps of this approach were identical to the first part of the "+" and "–" approach (3.6.2.1). The same amount of reticuloplasm (260 ml, 800 mg protein) was passed over MonoQ column and fractions containing FGM activity were pooled (32.5ml pool). In this approach we tried to reduce protein losses associated with surface adsorption by reducing the handling volumes and column matrix volumes as well. The MonoQ fractions pool was concentrated to 8 ml using Vivaspin concentrators at room temperature. The concentrated MonoQ pool was applied on a 4 ml scrambled peptide column equilibrated with the incubation buffer and incubated at 4°C overnight with rotation. The flow through of unspecific peptide column was directly applied on 2 ml (twice less than in the first approach) C69S affinity column equilibrated with the incubation buffer and incubated for 3 hours at 4°C with rotation. After collecting the flow through, both columns were washed with incubation buffer and incubation buffer containing 2M NaCl to remove unbound and weakly bound proteins and eluted with 4ml Laemmli buffer containing 1% SDS. The elution was performed by boiling the column matrices with Laemmli buffer at 95°C. The eluates were concentrated in the Vivaspin concentrator to 50 µl, boiled directly in the concentrator and applied on a 15% acrylamide SDS PAG. After separation of the proteins by SDS PAGE, the gel was stained overnight with Ruby Red Fluorescent dye to visualise the proteins (Fig. 3.28). For subsequent extraction of the bands and identification by mass spectrometry, the gel was incubated for 2-3 days in Roti-Blue Colloidal Coomassie solution. After extraction of the bands and in-gel tryptic digestion of the proteins the tryptic peptides were analysed by MALDI-TOF fingerprint (Reflex III, Bruker Daltonic), MALDI-TOF Post Source Decay (PSD) (Ultraflex, Bruker Daltonic), and NanoLC-ESI MS/MS (liquid chromatography combined with online electrospray-ionization tandem mass spectrometry, Ultimate/Esquire 3000+, Dionex, Bruker Daltonic).



MQ – MonoQ purified pool containing FGly generating activity
Fth 1 – flow through of the unspecific peptide column
Fth 2 – flow through of the ASA 65-80 C69S peptide column
Eluate 1 - SDS eluate of the unspecific peptide column
Eluate 2 - SDS eluate of the ASA 65-80 C69S affinity column
I.P. – proteins, identified by mass spetrometry
* - a 3000 fold larger aliquot of the final eluate was loaded on the gel as compared to the MonoQ eluate

Fig. 3.28. Elution of FGM-containing proteins from affinity matrix with SDS. FGM activity from 300g bovine pancreas was purified over MonoQ anion exchanger column, unspecific peptide column, and C69S affinity column. Bound proteins were eluted with Laemm´li buffer containing 1% SDS. The eluates were concentrated and directly used for separation of proteins on a 15 % acrylamide SDS-PAG (see text). Separated proteins were visualised by Ruby Red fluorescent dye. Comparison of the eluate of unspecific peptide column and affinity column revealed some protein bands present in the former and absent in the latter one.
We tried to identify all the bands present in the eluate of affinity column to estimate its complexity and protein composition.
Identified proteins:
1, 2, 4, 7 – Epstein-Barr virus nuclear antigen 2 co-activator
3 – analysis of this band revealed tryptic peptides belonging to 4 proteins: epoxide hydrolase (high significance, ER resident); molybdenum cofactor synthesis-step 1 protein; myosin light chain kinase and trifunctional enzyme (the latter is resident of mitochondrial matrix)
5 – two hypothetical proteins; amylase 2
6 – sorting nexin 6; molybdenum cofactor synthesis-step 1 protein; an unknown protein
8 – elastase 1
Other protein bands were either identified as human keratin or could not be identified.

Table 3.6.2.2 shows that the flow through of the affinity column still contains some FGM activity, since in this approach we significantly decreased the amount of affinity matrix. Nevertheless, 90% of the loaded activity is retained by the column.

Table 3.6.2.2. Recovery of FGM activity during purification

Purification steps	**Activity (% FGly modification per equivalent)**	**Recovery of FGM activity (% of loaded activity)**	**Recovery of FGM activity (% of reticuloplasm activity)**
Reticuloplasm	2.0	100	100
MonoQ purified pool	0.8	40	40
Unspecific column flow through	0.7	88	35
Affinity column flow through	0.06	9	3

Comparison of protein composition of the scrambled peptide column eluate and C69S affinity column eluate revealed some bands absent in the former and present in the latter one. We have tried to identify all the bands present in the SDS-eluate from the C69S affinity column, to estimate the protein complexity of the eluate. The keratin background in this gel was very high, which disturbed the analysis. Nevertheless, some protein bands could be identified (Fig. 3.28). This time we could not detect any protein belonging to the PDI family. Three of the identified proteins carry a signal peptide (epoxide hydrolase, an unknown protein and amylase 2 precursor). Molybdenum cofactor synthesis-step 1 protein is known as a cytosolic protein. However, a splice variant was detected carrying a signal peptide. Epoxide hydrolase and molybdenum cofactor synthesis-step 1 protein apart from PDI-proteins were considered as possible candidates to be involved in FGly modification (see Discussion).

4. Discussion

The aim of this work was to characterise the enzymatic machinery involved in the posttranslational modification of a critical cysteine residue in sulfatases to FGly and to establish a biochemical approach for purification and final identification of the proteins/cofactors involved.

4.1. The biochemical approach: enzyme source, substrate production and standard assay conditions

It was shown (Fey *et al.*, 2001) that FGly modification does not require membranes and is performed *in vitro* by reticuloplasm, i.e. luminal components of the endoplasmic reticulum. This reticuloplasm extracted from rough microsomes after perforation of the membranes with detergent is the starting material for the biochemical approach towards characterisation and identification of the FGly generating enzyme. The study involves characterisation of FGly generating machinery and purification of its components by different chromatographic protocols. For either purpose one would need defined assay conditions which make possible the measurement and quantification of FGly formation to allow for kinetic analysis on the one hand and for reliable estimation of activity loss and recovery after purification steps on the other hand.

Towards this goal we have established a standard FGM (FGly generating machinery) assay using a [^{35}S]methionine-labeled sulfatase polypeptide substrate that was translated *in vitro* by an mRNA-programmed reticulocyte lysate. Since FGly modification was shown to be independent of the membrane components, the assay substrate did not need the signal peptide, which was substituted by a heptapeptide providing a tryptic cleavage site, which was fused to an ASA sequence 64-158 with substituted methionines (Fig.3.1). This sequence contained amino acid residues essential for FGly formation (see Fig.1.5). To uncouple FGly modification from translation RNC technology was used, i.e. ribosome-associated nascent polypeptide chains were generated that remained ribosome-bound due to the lack of a stop-codon in the employed mRNA. This allowed to isolate the nascent sulfatase polypeptides from the translation system by sedimentation of ribosomes through a high-salt sucrose cushion. The polypeptides of these salt-washed RNCs were used as the substrate in the FGly *in vitro* generating assay with constant volume and incubation time. Puromycin, added during the assay, released the polypeptide chain from the ribosome.

The main advantage of the in vitro FGly modification assay was its high sensitivity, allowing to detect even very low amounts of FGly, since the last step – formation of FGly-hydrazone derivative shows practically no radioactive background (see Fig.3.3).
The chosen assay conditions allowed to measure modification of the substrate which was linear with time and amount of enzyme up to a turnover of 50% of substrate. FGM activity was calculated in „% FGly per microsomal equivalent“ units (see 3.1.3). This allowed to quantify FGly formation and to compare FGM activities determined under variation of parameters and to estimate losses and recovery of different purification protocols.
Using this standard *in vitro* FGly generating assay we compared FGM activity in different tissues using detergent solubilised microsomes, or reticuloplasm, from canine pancreas, bovine liver, testis, pancreas and adrenal glands. On the basis of the determined activities we have chosen bovine pancreas as the source for the FGly generating enzyme since it contained the most enriched FGM activity per g wet tissue, was obtainable in large amounts and its microsomal preparations were devoid of proteases.

4.2. Biochemical characterisation of the FGly generating enzyme

4.2.1. Enzymatic properties

Using the standard FGly modification assay we have identified several biochemical and kinetic parameters of FGM. Kinetic characterisation revealed typical enzymatic properties with a characteristic temperature dependence (activation energy 61kJ/mol) and strong inhibition by synthetic peptide comprising the modification motif (IC_{50} 135nM). A true K_m value could not be determined by saturation studies, because of the substrate limitation in the *in vitro* assay. At such low concentration of the *in vitro* synthesised polypeptide (approximately 15 fM) pre-steady state conditions may prevail with binding (and not catalysis) being the rate limiting step. A mutant peptide with cysteine 69 substituted by serine also had a strong inhibitory effect, although at approximately 5-fold higher concentration as compared to the peptide with native ASA sequence. The modifying machinery obviously recognises the modification sequence although serine cannot be modified to FGly neither *in vivo* nor *in vitro* (Dierks *et al*., 1997; Recksiek *et al*., 1998). Incubation with an ASA peptide where three critical residues of the FGly modification motif were scrambled (ASA65-80-C69P/P71R/R73C) did not have any inhibitory effect on FGM activity, indicating that FGly generating enzyme is not able to recognise the scrambled sequence.

pH optimum and pH stability studies of FGly formation showed that FGly generating machinery has a highly unusual alkaline pH optimum (10-10.5). On the other hand, its activity was irreversibly inhibited by pH values below pH 6. The hypothetical mechanism of oxidation of cysteine into FGly (Fig.1.2) proposes FGly formation in two steps: an enzyme mediated oxidation of the cysteine's thiol to a thioaldehyde group with subsequent hydrolysis which eliminates H_2S and yields FGly. This last hydrolytic step may be stimulated by OH^- ions and could be rate limiting at low pH. The irreversible inhibition of FGly formation by low pH, though, is not explained by this hypothesis.

4.2.2. Biochemical properties, cofactors involved

FGM activity shows slow kinetics *in vitro* (1% turnover in 20 min per equivalent of bovine pancreas microsomes), which could reflect substrate/cofactor limitation and/or, alternatively, may indicate a very low abundance of the modifying machinery in the reticuloplasm. Low abundance hypothesis is supported by the chromatographic data (see below). FGM shows some characteristics typical for an enzyme residing in the lumen of the ER. It contains glycoproteins, since it binds to Concanavalin A Sepharose (discussed below). It is stimulated by calcium ions (Fig.3.23) and sensitive to the SH/SS-redox milieu (Fig.3.20, 3.21). Unphysiologically high concentrations of GSH but also moderate concentrations of GSSG inhibited FGly formation. Thus, a possible role of GSSG as a cofactor that is directly involved in cysteine oxidation is highly unlikely. However, we cannot exclude that GSH/GSSG is required to maintain a redox potential favouring cysteine oxidation. A test of other cofactors which could theoretically be involved in cysteine oxidation did not bring any definite results.
Optimum conditions were observed at 2.5mM GSH or dithiothreitol indicating that these thiols are required to prevent oxidation of the enzyme cysteines. Adding 2.5mM DTT to the buffers during chromatography significantly increased recovery and stability of FGM activity in the purified fractions (Fig.3.24). The substrate's cysteine obviously was not affected in these experiments, because only pretreatment of the purified fractions with dithiothreitol led to stimulation of FGM activity, whereas pretreatment of RNCs with dithiothreitol had no or partial inhibitory effect (Fig.3.22). Passing the reticuloplasm through a gel filtration column and also long term dialysis did not reduce its specific modifying activity (in the presence of dithiothreitol and calcium ions) suggesting that no diffusible low molecular weight cofactor is required for *in vitro* FGly formation. The significant stimulation of activity in a MonoQ-purified fraction by boiled reticuloplasm (Fig.3.19), though, suggests that a heat stable

cofactor associated with proteins could be involved in FGly formation. Another possible explanation of this phenomenon is that the exposed SH-groups of the denatures proteins can prevent oxidation of the enzyme's cysteines. Detailed investigation of this question is a matter of further studies.
Salt sensitivity tests showed that the enzyme can tolerate treatment with high salt concentrations (1.5M NaCl or 2M $(NH_4)_2SO_4$) with about 50% to 60% activity recovery when salt was present during the assay. After removal of salt the modification activity was 100% restored. Incubation with high concentrations of chaotropic salt (2M KSCN) totally inhibited the enzyme activity during incubation with the substrate. In this case 50% of the activity could be recovered after salt removal.

4.3. Single chromatographic protocols for partial purification of FGM activity

In this study we established several purification protocols for partial purification of FGM activity. The overview of their efficiencies, advantages and disadvantages is given in Table.4.1.

Table 4.1. Efficiencies of different chromatographic protocols with respect to purification of FGM activity

Type of chromatography	*Column/System*	*FGly activity recovery (% of loaded activity)*	*Advantages*	*Disadvantages*
Anion exchange chromatography	MonoQ® PC 1.6/5 (0.1ml)/SMART	40-60	Reproducibility, high resolution, mild elution conditions	Low capacity, only for analytical scale
	MonoQ® HR 5/5 (1ml)/SMART	40-60	Reproducibility, high resolution, mild elution conditions	Average capacity
	MonoQ® HR 10/10 (8ml)/SMART	40-60	Reproducibility, high resolution, mild elution conditions, very high capacity of the column	Difficult handling (loading of the big volumes by super-loop, refilling of the pumps required)
Cation exchange chromatography	MonoS® PC 1.6/5 (0.1ml)/SMART	1-10	Mild elution conditions	Low capacity. Requires pH 5.5 in the buffers, which inactivates the

				enzyme irreversibly
Gel filtration chromatography	Superdex® 200 PC 3.2/30 (2.4ml)/SMART	40-60	Does not require buffer exchange, mild elution conditions, good resolution, good reproducibility	Low capacity of the column. Low sample application volume, dilution during chromatography
Hydrophobic interaction chromatography	Phenyl Superose® PC 1.6/5 (0.1ml)/SMART	20-40	Good reproducibility	Sample should be applied on the column in high salt buffer which has inhibitory effect on FGM activity. Low capacity
	POROS® 20 PE/ Vision Workstation	20-40	Fast purification, unlimited capacity (column size can be changed)	Very high degree of fractions dilution. Low resolution, low reproducibility
Lectin chromatography	Concanavalin A Sepharose	20-40	Easy handling, mild elution conditions, allows FGM concentration	Low binding specificity (binds many of ER proteins)
Heparin chromatography	Heparin sepharose	40	Good activity recovery, mild elution conditions	Low binding specificity
Dye affinity chromatography	See. 3.4.7	0-15	Easy handling, mild elution conditions	Low recovery, low binding specificity
Chromatography on affinity matrices with immobilised peptides	ASA 65-80	0	Easy handling, high specificity of the binding	No recovery of activity
	ASA 65-80 C69S	10-40	Easy handling, high specificity of binding, high capacity	Very strong binding, elution requires harsh conditions or inhibitory peptide

	ASA 65-80 „scrambled“	0 (activity not bound)	Does not bind FGM activity, can be used to „pick up“ contaminating chaperones and other peptide-binding proteins	-

As shown in table 4.1, the majority of established single purification protocols provided reasonable recoveries of FGM activity. One of the most efficient methods for purification of FGM activity is chromatography on MonoQ-material. All three MonoQ columns used provided reproducible and efficient separation of reticuloplasm proteins with 40-60% recovery of FGM activity when used as the first purification step. The MonoQ® PC 1.6/5 and MonoQ® HR 5/5 columns are ideal for analytical experiments and purification on a small scale. Very high capacity of MonoQ® HR 10/10 column made it very suitable for big scale purifications, allowing to remove up to 97%-99% of proteins in single step (Fig.3.7). pH optimisation studies for MonoQ columns (Fig.3.6) showed that the best purification one can perform on a MonoQ® HR 10/10 column at pH 8.0. The cation exchange column is not suitable for purification of FGly generating enzyme in the native state, since the buffers need to have a pH of 5.5 or lower, which affects FGM activity irreversibly. Hydrophobic interaction and gel filtration chromatography provide good activity recovery and are good options for purifying FGM activity at the analytical scale. Lectin and heparin chromatography provided low specificity of binding, though these chromatographic media could be a good choice for big scale purification, since they can be used in a batch procedure and, thus, will not impose any size and capacity restrictions.

Affinity purification on immobilised substrates is one of the most specific ways to isolate proteins. We created two affinity matrices with immobilised peptides ASA 65-80 (C69 column) and ASA 65-80 C69S (C69S column). Passing the reticuloplasm over these affinity columns resulted in depletion of FGM activity. In case of the C69S affinity column we were not able to detect FGly generating activity neither in the flow through nor in the eluate. Surprisingly, the activity could be eluted from C69S column with much better recovery (40% in best cases). Elution could be performed either with 2M KSCN or with 25µM ASA 65-80 C69S peptide. Salt elution was not preferable as 2M KSCN was shown to have partial inhibitory effect on FGM activity. The peptide elution was chosen for big scale purifications

since it is more specific, applies less harsh conditions and the peptide can be easily removed from the eluate by dialysis.

There are several possible explanations of the phenomenon that the FGM activity could be eluted only from the mutant-peptide affinity column and not from the column with the native ASA peptide. It is possible that the modifying enzyme converts the cysteine 69 of the immobilised ASA 65-80 peptide into FGly during incubation with affinity matrix. If after modification the enzyme is reduced and needs a certain cofactor to be re-oxidised to the active state and if this cofactor is limiting in the *in vitro* assay, the enzyme would remain in inactive form. However, reconstitution experiments, i.e. combination of the affinity column eluate and the flow through, did not lead to activity recovery (not shown), what makes this hypothesis unlikely. Another possible explanation is that binding to the native form of the peptide is so strong that we were not able to elute bound protein even under very strong desorbing conditions. To test for this hypothesis we subjected reticuloplasm to incubation with peptides ASA 65-80 and ASA 65-80 C69S (at a concentration of 25µM) for 2 hours on ice and then dialysed overnight to remove the peptides. After this treatment the activity of reticuloplasm was recovered by 50-80% (ASA 65-80) and 75-100% (ASA 65-80 C69S), indicating that binding of FGly enzyme to both peptides is reversible. However, since the local peptide concentrations on the column may be very high it may well be that separation of enzyme-peptide interaction on the column and in the eluate is very inefficient. Therefore, the C69 affinity column may be washed thoroughly and finally eluted with SDS to obtain strongly bound FGM protein(s).

The fact that FGly enzyme could be eluted from the C69S affinity matrix only with very high concentration of chaotropic salt or with the substrate peptide might mean that binding of the substrate to the catalytic centre of the enzyme is very strong, even when the cysteine 69 residue is substituted by serine, which can not be modified to FGly. One of the ways to decrease the strength of enzyme binding to the catalytic centre is to introduce other amino acids on the place of cysteine. Possible weaker binding of the enzyme to these mutant peptides may result in better recoveries of activity in the eluate.

4.4. Purification strategies and scaling up

Although many of the single purification protocols provided good to reasonable recoveries of FGM activity, combination of two or more of these protocols in most cases led to total loss of activity/protein (not shown). Optimisation studies showed that in some cases loss of activity

was associated with instability of FGly generating enzyme in the partly purified state. After addition of dithiothreitol (2.5mM) and calcium ions (15µM) to the chromatographic buffers the stability of purified fractions significantly increased (Fig.3.24). Another problem was severe protein loss in highly purified fractions due to dilution and absorption to surfaces. This problem could be solved by addition of carrier proteins (BSA or cytochrom c) to elution buffers and ultrathimbles used for dialysis and concentration of affinity eluates (see Table 3.5.5). A mix of 20 amino acids at final concentration of 1mg/ml, added to elution buffer during purification on peptide affinity columns significantly improved protein and activity recovery and was chosen for further purification protocols, since amino acids did not introduce protein contaminations which would interfere with the following identification of purified proteins.

Combinations of different columns in various sequence were tested leading to the conclusion that the optimal purification protocol should include a minimal number of most efficient purification steps to avoid unnecessary handling, which would lead to protein and activity losses. The two most efficient chromatographic procedures chosen were MonoQ and peptide affinity columns. The former provided good reproducibility, recovery and mild elution conditions and the latter was the most specific for binding of FGM activity. The MonoQ was the most efficient as the first purification step, because it removed approximately 80% (MonoQ® PC 1.6/5 and MonoQ® HR 5/5) to 99% (MonoQ® HR 10/10) of reticuloplasm proteins from the fraction containing FGM activity. The other advantage of this chromatographic step was concentration of FGM activity 2-6 fold as compared to reticuloplasm.

The first purification protocol described in this study involved purification on MonoQ® PC 1.6/5 column and C69S peptide affinity column with following rechromatography of the active eluate on the affinity column. The rechromatography step was introduced to decrease the background of proteins which unspecificly bind to the affinity matrix. 120 mg reticuloplasm proteins of microsomes prepared from 50g of pancreas was used, the activity recovery in the final eluate was 9% of that of the reticuloplasm. Protein bands of the final eluate were analysed by in-gel digestion and mass spectrometry. Some bands from the final eluate could not be identified and almost all identified bands belonged to the PDI protein family (Fig.3.26). It is well possible that a PDI enzyme and chaperones are involved in FGly modification. However, these proteins are known for their high affinity to peptides in general

and it is therefore also possible that these proteins were unspecificly bound to the peptides presented on the affinity matrix.

To reduce unspecific binding in the next purification strategy we introduced a peptide column with ASA "scrambled" sequence peptide as an intermediate step in the purification protocol. Proteins which bind to the peptide affinity column unspecificly should bind with the same efficiency to the scrambled peptide, which does not retain the FGly generating enzyme (see Table 3.4.7.1). As the first purification step we used the MonoQ® HR 10/10 column, since it provided high capacity and a higher purification efficiency than the other two anion exchange columns. Purification of FGly enzyme from the same amount of reticuloplasm proteins (120 mg protein, corresponding to 50g pancreas) resulted in an active final affinity active eluate with 10% recovery of the starting activity. However, after separation of purified proteins by SDS PAGE and staining of the gel with Coomassie blue there were no protein bands visible. To be able to load the whole final active eluate on the gel we had to introduce a concentration step (the sample was concentrated in Vivaspin concentrator and boiled with Laemmli buffer directly in the concentrator), which could also lead to protein losses. We concluded that the amount of starting material was not sufficient to purify the proteins in the amount necessary for their identification. As it was pointed out before, the FGly generating enzyme obviously belongs to the very low abundant proteins of the cell. The purification protocol needed to be scaled up several fold.

The two final purification strategies described in this study were developed to purify FGM activity from 800 mg protein reticuloplasm proteins extracted from microsomes of 300g bovine pancreas (Fig.4.2). The first part of both strategies is the same – purification on MonoQ, scrambled peptide column and C69S affinity peptide column. In case of the "+ and - approach" the eluate from affinity column was dialysed to remove peptide used for elution and split into 2 halves. The first half was concentrated and loaded directly on the gel (the "+" lane, containing FGM activity). The second half was depleted of FGM activity by incubation with C69S peptide affinity matrix and the flow through of this column then was also applied on the SDS PAG (the "-" lane, containing no FGM activity). The advantage of this approach is that one could directly compare two lanes and identify the bands present in "+" lane and absent in "-" lane. The disadvantage is that during elution and dialysis one has unavoidable unspecific losses of protein, since the protein concentration in the eluate is very low. For that reason we designed the second approach where after binding of FGM activity to the affinity peptide column all bound proteins were eluted at 95°C with Laemmli buffer (containing 1%

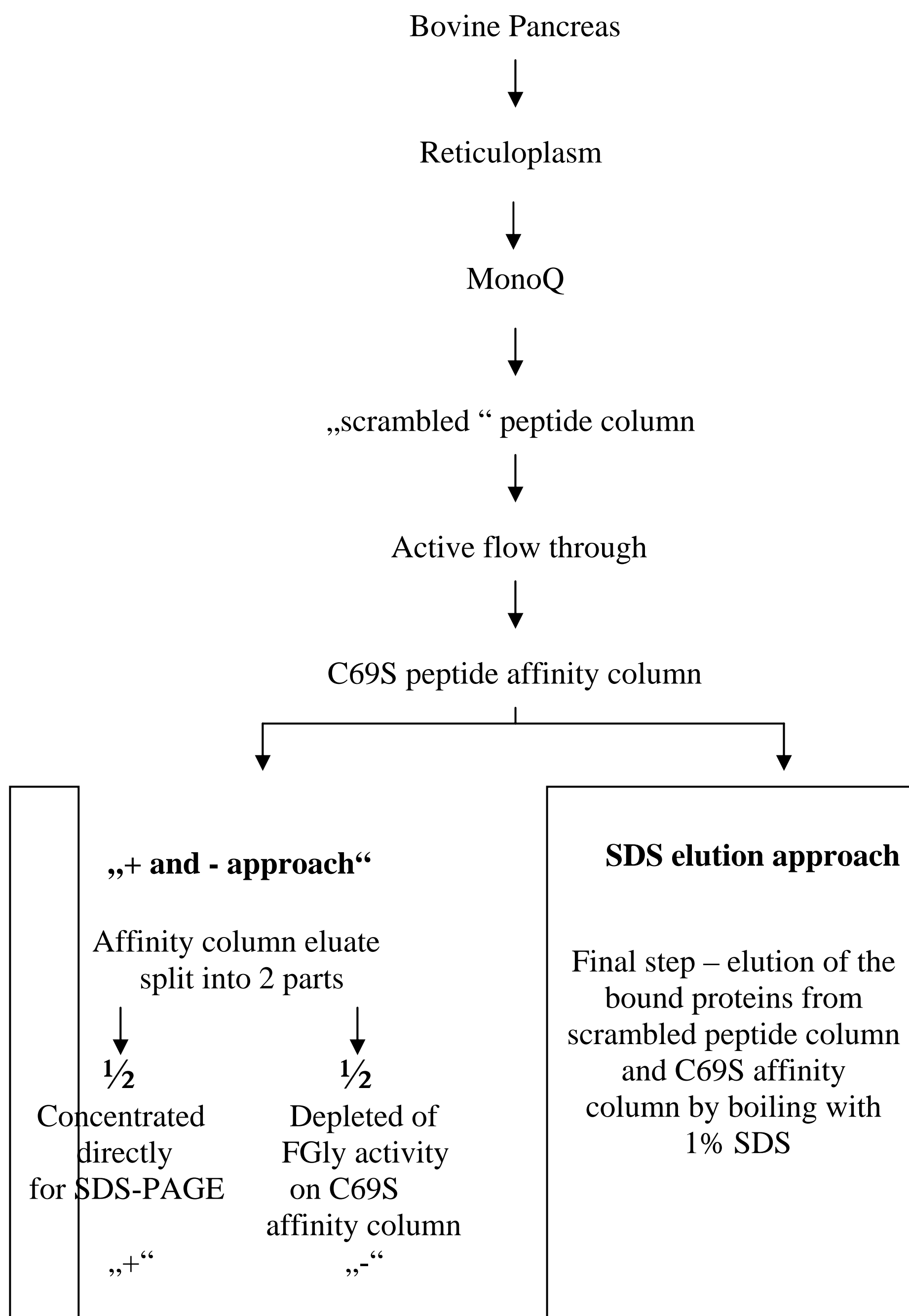


Fig.4.2. Strategies for purification of FGly generating activity on a big scale

SDS). At such concentration of SDS all bound proteins should be effectively eluted from the column matrix and, besides, SDS should prevent unspecific binding of proteins to the surrounding surfaces. Of course, measurement of FGly generating activity in the SDS eluate was not possible. We supposed that at that elution conditions recovery of bound proteins would be maximal and unspecific protein loss would be minimal. Indeed, the amount of protein present in the final SDS eluate in the second approach was sufficient to identify more

proteins than in case of “+ and - approach” (compare Fig.3.27 and Fig 3.28). Though both strategies do not lead to purification of FGly generating enzyme to homogeneity, they provide a fast and effective way to get the most pure and FGM-enriched eluate on a big scale. The best results one would get by comparing data obtained from both approaches.

4.5. Candidate proteins on the role of FGly generating enzyme

It was shown recently that an iron-sulphur protein AtsB is essential for FGly formation in serine-type sulfatase in bacteria *Klebsiella Pneumoniae* (Szameit *et al*., 1999; Marquordt *et al*., 2002) AtsB is located in the cytosol and is interacting directly with the serine-type FGly modification motif, which allows to consider the possibility that AtsB itself is the oxidising enzyme converting serine to FGly. There are no serine-type sulfatases in eukaryotes and only very weak homologs of AtsB are encoded in mammalian genomes, the best one being viperin (Chin and Cresswell, 2001). Viperin localises to the lumen of the endoplasmic reticulum and shows 26% identity with the *N*-terminal third of AtsB and thus, could be a possible candidate for the role of FGly generating enzyme. We tested by Western-blotting, whether viperin is present in the partly purified FGM activity containing fractions. Although viperin was present in the MonoQ purified FGM containing fraction, it obviously was not retained by the C69S affinity column, since it could be detected in the FGM-activity depleted flow through (not shown). Therefore, viperin is not likely to be a part of FGM.

Purification strategies for partial purification of FGM activity developed in this study led to identification of some proteins which could be possible candidates on the role of FGly generating enzyme. In the eluates from affinity columns we could detect several members of the PDI protein family, namely: ERp57, PDI, PDIp and CaBP1. ERp57 was present in the active eluate of the two independent purification approaches (Fig. 3.26 and Fig.3.27). Most, if not all members of the PDI family have both enzymic and chaperone function to fullfill in the ER and are believed to be redox-active, although the latter is not yet experimentally confirmed for all proteins (Ferrari and Söling, 1999). Most have been shown to catalyse the reduction of disulphide bonds *in vitro* and *in vivo*. One could consider PDI for the role of FGly generating enzyme, as it is localised to the ER and has the redox activity. However, all proteins of the PDI family identified in this study are abundantly expressed in the cell, which would contradict with our observation that FGly-generating enzyme is not an abundant protein. Besides, members of the PDI family show high affinity to the peptides (as well as calreticulin and GRp94), what could explain their co-purification on the peptide affinity

column. But we can not exclude that a member of the PDI protein family or other chaperone is involved in FGly formation, since its redox activity can help to maintain the FGly generating enzyme in the oxidised/active state.

Last approach with SDS elution of the affinity column led to identification of quite a number of different proteins (Fig.3.28), among them two hypothetical proteins (have apparently no signal sequence) and an unknown protein which has a signal sequence and one N-glycosylation site, but no ER-retention signal. This unknown protein shows no sequence homology to any other protein. Out of other identified proteins we consider only two to be of interest: epoxide hydrolase and molybdenum cofactor synthesis-step 1 protein. Epoxide hydrolase is localised to the ER and is involved in the hydrolysis of various epoxides and epoxide intermediates (Hassett et al., 1989). It is unlikely to be a FGly generating enzyme since it has no oxidative function, but as a hydrolase, it may be a part of FGly-generating complex and catalyse the formation of an aldehyde hydrate, which is the last step of oxidation of cysteine to FGly (see Fig.1.2). Epoxide hydrolase catalyses an addition of a water molecule to the epoxide with a formation of dihydrodiol (Fig.4.3*B*), so it could theoretically also catalyse hydrolysis of an aldehyde (Fig. 4.3*A*).

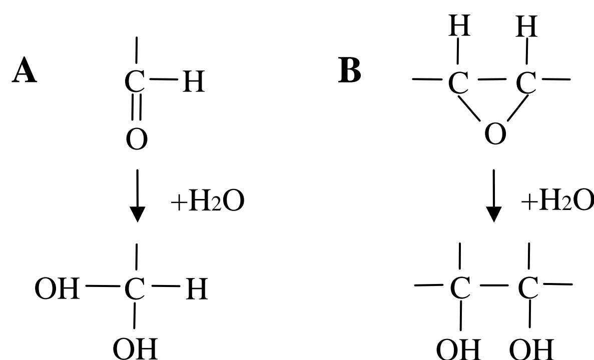


Fig. 4.3. Formation of an aldehyde hydrate and hydrolysis of an epoxide. *A*. Addition of a water molecule leads to formation of a geminal diol of an aldehyde hydrate. *B*. Hydrolysis of an epoxide group with the formation of a dihydrodiol.

A molybdenum cofactor synthesis-step 1 protein (MOCS1) is a cytosolic protein, although there exists an MOCS1 ubiquitously pressed variant with alternatively spliced Exon1, carrying a signal peptide. Alternative splicing of the MOCS1 locus results in expression of several forms of the protein: MOCS1A, MOCS1B and MOCS1A-B. The fact that MOCS1A,

and therefore also MOCS1A-B are the iron-sulphur proteins (Reiss *et al.*, 1998) makes an interesting connection to the iron-sulphur protein AtsB, involved in FGly formation in serine-type sulfatases in bacteria.

The data on the candidate proteins obtained in this work is still preliminary, as many of the proteins could be identified only in one of the undertaken purification approaches. Further studies and combination of different approaches should narrow the list of possible candidates.

4.6. Biochemical approach limitations

The biochemical approach for purification of FGly generating machinery undertaken in this study has some limitations which influence its speed and efficiency. The first and foremost is the limitation connected with the very low abundance of FGM in the cell. One should start the purification procedure with very high amounts of starting tissue, in this case bovine pancreas. The microsomes must be prepared from the fresh pancreas at one day, otherwise the FGM activity drops significantly. We have tested whether pancreas can be shock frozen and kept at –80°C, but the results showed that microsomes prepared from the frozen pancreas have less specific FGM activity than the ones prepared from fresh pancreas. Tests of microsomes prepared from bovine testis showed that testis has several-fold more FGM activity if expressed in activity units per ml reticuloplasm, though less than pancreas if expressed in activity units per g wet tissue (see Results, Table 3.2). The advantage of testis is that it can be kept on ice for one day without significant FGM activity loss, that allows preparation of microsomes on a bigger scale than in case of pancreas. Thus, testis in the future could be the better protein source for purification of FGly generating enzyme, since FGM specific activity is much higher in testis reticuloplasm compared to pancreas reticuloplasm.

The next limiting factor is the recovery of FGM activity after purification of reticuloplasm proteins on more than two columns. Recovery was very low and sometimes the activity was barely detectable. Losses of activity are connected with thermal inactivation, protein losses and, probably, loss of a specific component/cofactor during long purification steps. Thus, one should use the fastest and most efficient purification protocol to be able to detect activity in the last purified fraction. One should also find the way to concentrate the final active fraction, which in case of affinity purification is usually very diluted, to be able to load it as a whole on the SDS gel. This step is very important, as, considering the dilution and very low protein concentration in the last eluate, losses due to absorption to surfaces in this case can be dramatic.

One of the very important limiting factors was the identification step involving SDS-PAGE, protein visualisation, in-gel tryptic digestion and mass spectrometry. Even the fastest purification protocol requires a lot of handling of the purified material, which often results in contaminations, the most common of which is contamination with human keratin. Considering that FGly generating enzyme is present in very low amounts, contaminating keratin may mask the purified protein and interfere with following mass spectrometrical analysis. The other obstacle connected with identification is the database for bovine proteins, which is far less well developed than the databases for human or mouse.

4.7. Outlook

Although the biochemical approach has proven to be one of the fastest and most efficient approaches for purification and characterisation of proteins, to identify the FGly generating enzyme it alone may not be sufficient. Apparent very low abundance of FGly generating enzyme in mammalian cell makes the direct purification approach not optimal due to severe activity and protein losses after several purification steps. One would possibly need to include other approaches and to combine them with the developed biochemical approach.

One of the possible ways is a combination of the biochemical and a proteomics approaches. The anion exchanger MonoQ® HR 10/10 (8ml) provides good recoveries of activity and allows purification of FGM activity on a big scale. The 2-dimensional gel electrophoresis of the purified MonoQ fraction with following identification of proteins could provide the information about the actual complexity of the active fraction. If such an active MonoQ eluate would be depleted of FGM activity by incubation with the peptide affinity column, the 2-D gel of the inactive flow through could be compared to the 2-D gel of the active MonoQ fraction. Proteins that are missing in the inactive affinity flow through gel would be good candidates on the role of FGly generating enzyme. One could decrease unspecific binding of proteins, especially chaperones, by introducing the unspecific peptide column as an intermediate step of purification, as it was done in purification protocols developed in this work. The main advantage of this approach is that one should start with much less purified material and thus significant protein losses would be avoided. A possible disadvantage of the approach may be that if FGly generating enzyme is indeed a very low abundant protein, it can be masked on a gel by other proteins present in high amounts, like chaperones.

Another approach, widely used for protein identification, could be a chemical crosslink approach. A chemical crosslinker group, coupled to ASA 65-80 native or mutant peptide and

photo-activated during interaction of the catalytic center of FGly generating enzyme with the peptide would introduce a covalent bond between peptide and enzyme. A radioactively labelled or biotinylated peptide as an affinity ligand would allow to visualise the crosslinked protein after SDS PAGE. One of the very efficient methods of photochemical crosslinking is introducing benzophenone photoprobes in the enzyme substrates, including peptides (Prestwich *et al*., 1997). This photoaffinity labels were successfully used for identification of binding sites in oxidative enzymes (Andersen *et al*., 1995; Denner-Ancona *et al*., 1995). An approach with a radioactively labelled crosslink, after reaction with a partially, e.g. MonoQ-purified FGM fraction, would provide information about the size and complexity of the enzyme machinery, which would narrow the target group of possible candidates. Once introduced, the affinity label could be traced during further chromatographic steps, like purification on a peptide affinity column or gel filtration column, or during 2D gel electrophoresis without further need for FGM activity determination.

A completely different possibility to identify FGly generating enzyme is to use a GAL4 yeast two-hybrid system to identify proteins interacting with sulfatase fragment carrying the FGly modification motif. One could use the human genomic library as „prey“ and a sulfatase polypeptide as a „bait“. Usage of the full length sulfatase polypeptide as a bait would probably make no use, since FGly modification occurs during or shortly after translation when the cysteine residue is easily accessible to the modifying enzyme. As a control one could use the sulfatase polypeptide with scrambled FGly modification sequence, this would help to detect the unspecificly binding proteins, similarly as done in the affinity purificaton approach. Of course, the best result one would get by comparison of candidate proteins identified by 2-hybride and the biochemical approach.

Whatever approach is used for identification of candidates for the role of FGM, all these candidate proteins need to be verified. The possible ways for verification are: a knock-out experiments in cell culture (RNAi knock-out), allowing to “knock-out” a certain gene; transfection of MSD-fibroblasts with candidate protein’s cDNA; or sequencing of the corresponding genes in MSD patients, looking for mutations. A combination of these approaches would provide the most certain result.

5. Abstract

Sulfatases carry at their active site a Cα-formylglycine (FGly) residue that is essential for enzyme activity. The formyl group of this FGly is directly involved in sulfate ester cleavage. A defect in FGly formation in human is the cause of a recessively inherited disease called Multiple Sulfatase Deficiency, characterised by the synthesis of the catalytically inactive sulfatase polypeptides and by the accumulation of their unprocessed substrates in the lysosomes. FGly is generated by oxidation of a concerved cystein (pro- and eukaryotes) or serine residue (prokaryotes) comprised in the sequience motif C/S-x-P-x-R. In eukaryotes the modification is catalysed by lumenal components of the endoplasmic reticulum during or after protein translocation and prior to folding of sulfatases. Under *in vitro* conditions, using an enriched and soluble protein fraction, FGly formation could be observed under strictly posttranslational conditions and independent of a signal peptide. The modification reaction was characterised kinetically and with respect to cofactor requirement, albeit the acceptor of the reducing equivalents during cystein oxidation remains unknown.

So far the enzymatic machinery involved in FGly modification could not be identified. We tried to purify it from the reticuloplasm of bovine pancreas microsomes using a number of chromatographic techniques with the following identification of proteins. We established several chromatographic protocols for separation of luminal proteins on different columns (ion exchanger, gel filtration, hydrophobic interaction, lectin chromatography, affinity chromatography etc.). FGly generating enzyme was characterised kinetically and biochemically. By combination of chromatographic protocols we purified the FGly generating activity and identified the bands visualised after separation of proteins by SDS PAGE. Several possible candidates on the role of FGly generating enzyme are identified.

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7. Abbreviations

AA	Acrylamide
Amp.	Ampicilline
APS	Ammonium peroxide sulfate
ASA	Arylsulfatase A
ASB	Arylsulfatase B
Aa	Amino acid
Arg	Arginine
ATP	Adenodin-5'-triphosphate
bAA	Bisacrylamide
bp	Base pairs
BPB	Brom phenol blue
BSA	Bovine serum albumin
^{o}C	Grade Celsius
cDNA	Complementary DNA
Ci	Curie
ConA	Concanavalin A
cpm	Impulse per minute
C69	ASA 65-80 peptide
C69S	ASA 65-80 C69S peptide
Da	Dalton
DTT	Dithiothreitol
Dist.	Distilled
ddH_2O	Distilled H_2O
DMSO	Dimethylsulfoxid
DNA	Desoxyribonuclein acid
DNPH	Dinitrophenylhydrasine
dNTPs	2'-Desoxyribonucleoside-5'-phosphate
E	Extinction
E.coli	*Escherichia coli*
EDTA	Ethylendiamintetraacetat
eq	equivalents
ER	Endoplasmic reticulum

et al.	*et alteri* (and others)
FGly-residue	Formylglycine residue
g	gramm
Gly	Glycine
GuHCl	Guanidinhydrochloride
h	hour
HPLC	High perfomance liquid chromatography
His	Histidine
Ig	Immunoglobulin
k	Kilo, -(x10^{-3})
kb	kilobase
K.pneumoniae	Klebsiella pneumoniae
l	liter
LB-medium	Luria-Broth medium
m	meter
M	molar
µ	micro, -(x10^{-6})
mA	milliamper
min	minute
$MgCl_2$	Magnesium chloride
MSD	Multiple sulfatase deficiency
mRNA	messenger RNA
n	nano, -(x10^{-9})
OD	Optical density
p.a.	pro analysis (for analysis)
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphat-buffered solution
PCR	Polimerase chain reaction
PDI	Protein disulphide isomerase
pNCS	para-nitrocatecholsulfate
pNPS	para-nitrophenyllsulfate
P.aeruginosa	*Pseudomonas aeruginosa*
RNA	Ribonucleic acid
RNAse	Ribonuclease

RP	Reversed phase
rpm	Rotation per minute
RT	Room temperature
rRNA	Ribosomal RNA
s	secund
^{35}S	Sulphur 35
SDS	Sodium dodecyl sulfate
t	time
T	thymin
Taq	Thermophilius aquatics
TAE	Tris-acetat-EDTA-buffer
TBS	Tris-buffered solution
TCA	Trichlor-acetic acid
TGN	*trans*-Golgi-network
TE	Tris-EDTA
TEMED	N,N,N,N-tetramethylethylendiamin
Tris	Tris-(hydroxymethyl)-aminomethan
U	Uracil, Unit
UV	Ultraviolet
V	Volt
v/v	Volume pro volume
wt	Wild type
w/v	Weight pro volume

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Publications and conferences

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2. European Science Foundation Conference on Protein Targeting. 28th September – 3rd October 2001, Acquafredda di Maratea, Italy, poster

Lebenslauf

Am 5 April 1972 wurde ich als erstes Kind von Tatiana Nikolaevna Borissenko, geb. Kotchubei, und Viktor Semionovitch Borissenko in Sochi (Gebiet Krasnodar, Russland) geboren. Ich besitze die russische Staatsangehörigkeit. Von September 1979 bis Juni 1982 besuchte ich die Grundschule Nummer 4 in Sosnoviy Bor (Gebiet Leningrad, Russland). Ab September 1982 war ich Schülerin des Gymnasiums Nummer 6 in Sosnoviy Bor. Meine Schulausbildung schloss ich dort im Juni 1989 mit dem Abitur ab.

Im Wintersemester 1990 begann ich das Studium der Biologie an der Staatsuniversität zu Sankt-Petersburg. Meine Magisterarbeit im Fachbereich Gehirn Physiologie begann ich im September 1995. Die Abgabe der Magisterarbeit mit dem Titel „Charakterisierung der Sprechen des Taubes Kinder“ erfolgte im Oktober 1996. Anschließend fand die mündliche Diplom-Hauptprüfung im Oktober 1996 statt.

Ab Januar 1996 bis April 1999 habe ich ein weiterführendes Praktikum in der Fächern Physiologie und Molekularbiologie im Physiologische Institut der Russische Akademie der Wissenschaften, Sankt-Petersburg durchgeführt.

Im Juli 1999 begann ich in der Abteilung von Biochemie II der Universität Göttingen unter der Anleitung von Herrn Dr. T. Dierks den experimentellen Teil der Dissertation mit dem Thema „Posttranslational generation of Cα-formylglycin in eukaryotic sulfatases: development of the biochemical approach for the characterisation and purification of the modifying enzyme“.