

Conversion of cysteine to formylglycine in eukaryotic sulfatases occurs by a common mechanism in the endoplasmic reticulum

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Received 18 December 1997

Abstract Sulfatases undergo an unusual protein modification leading to conversion of a specific cysteine residue into α -formylglycine. This conversion is essential for catalytic activity. In arylsulfatase A the α -formylglycine is generated inside the endoplasmic reticulum at a late stage of protein translocation. Using *in vitro* translation in the presence of transport-competent microsomes we found that arylsulfatase B is also modified in a similar way by the formylglycine-generating machinery. Modification depended on protein transport and on the correct position of the relevant cysteine. Arylsulfatase A and B did not compete for modification, as became apparent in co-expression experiments. This could argue for an association of the modification machinery with the protein translocation apparatus.

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Key words: Sulfatase; Endoplasmic reticulum; Protein modification; Protein transport; Multiple sulfatase deficiency

1. Introduction

In eukaryotic sulfatases a α -formylglycine (FGly, 2-amino-3-oxo-propanoic acid) is found at a position where the genes encode a cysteine [1,2]. Deficiency of the FGly is associated with catalytic inactivity of the sulfatases, as is found in multiple sulfatase deficiency, a rare inherited human lysosomal storage disorder [1,3,4]. The FGly residue is part of the catalytic site, as has been shown by crystallographic analysis of two lysosomal sulfatases, arylsulfatase A (ASA) and arylsulfatase B (ASB) [5,6]. The aldehyde group of the FGly residue, most likely in its hydrated form [5,7], serves as an acceptor for sulfate during sulfate ester cleavage [5–7]. In acting as a geminal diol the FGly hydrate allows for efficient ester hydrolysis at the acidic pH of lysosomes. The catalytic mechanism involves trans-esterification of the sulfate group from the substrate to the first hydroxyl, from where it is eliminated due to the presence of the second hydroxyl [5,7].

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Abbreviations: ASA, arylsulfatase A (cerebroside-3-sulfate 3-sulfohydrolase, EC 3.1.6.8); ASB, arylsulfatase B (*N*-acetylgalactosamine-4-sulfate 4-sulfohydrolase, EC 3.1.6.9); DNP, 2,4-dinitrophenyl; ER, endoplasmic reticulum; FGly, C α -formylglycine; P3, cysteine-containing form of tryptic peptide 3 of ASB; P3*, FGly-containing form of tryptic peptide 3 of ASB; P3C, C-terminal fragment of P3 generated by endopeptidase AspN; P3*C, C-terminal fragment of P3* generated by endopeptidase AspN; RP-HPLC, reversed phase-high performance liquid chromatography

The FGly residue in ASA is generated by protein modification at a late stage of co-translational protein translocation into the endoplasmic reticulum (ER), as could be shown by *in vitro* synthesis and translocation of nascent ASA polypeptides into canine pancreas microsomes [8]. The modification is directed by a linear sequence of 16 residues surrounding the cysteine to be modified [8]. This sequence includes an X-P-S-R and an L/M-T-G-R/K tetrapeptide, which are located C-terminal of the cysteine and which are conserved among all human sulfatases [2,9].

Since in multiple sulfatase deficiency the activity of all tested sulfatases was severely decreased, it is likely that the microsomal modifying machinery acts on all newly synthesized sulfatase polypeptides. So far nothing is known about the modifying machinery and its components, which are supposed to catalyze a two-step reaction involving an oxidation of the thiol to a thioaldehyde group and a hydrolytic release of H₂S [1]. To establish whether all sulfatases are modified in the ER and to qualify the *in vitro* translation/translocation system as a biochemical means to characterize the modifying machinery we determined *in vitro* the modification of ASB and compared it to that of ASA.

2. Materials and methods

2.1. Site-directed mutagenesis

Mutagenesis of a cDNA coding for human ASB [10] was carried out by the QuikChange method (Stratagene) using complementary primers (coding sequence GTGCTCCTGGACATGTACTACACG-CAG) that substituted the Asn codon 84 by a methionine codon. Inversion of codon 91 (Cys) and 92 (Thr) was achieved by PCR using a non-coding primer (CTGGTAGCGGCCAGTGAGCAGCTGG-CTCCGCGACGGGCAGGTCAGCGGCTGCG) covering the nearby *XcmI* site. The PCR product was subcloned as a *HindIII/XcmI* fragment replacing the corresponding fragment of the template DNA pRL2 (see below). No Pfu- or Taq-polymerase errors were detected upon sequencing of the entire coding sequences.

2.2. Protein expression and purification

The cDNAs of wild-type ASB and ASB-N84M, respectively, were cloned as *BamHI/EcoRI* fragments into the pMPSVHE vector [11] downstream of the myeloproliferative sarcoma virus promoter. The resulting plasmid and PGK-hygro as selection marker were used for stable transfection of mouse embryonic fibroblasts deficient in both mannose 6-phosphate receptors, as described [12]. The expressed ASB protein was purified from the secretions of the cells by affinity chromatography [13]. The specific activity of both wild-type and mutant enzyme was similar (about 100 U/mg). Synthesis of the ASB proteins in an immature form (64 kDa), that due to constitutive secretion from the mannose 6-phosphate receptor-deficient cells circumvent lysosomal processing [14], did not affect the catalytic activity, since mature ASB expressed in BHK cells had the same sulfatase activity. The expressed proteins carried exclusively FGly and no cysteine in position 91, as was verified by mass spectrometry (see below). Expression and purification of ASA-F59M protein was described earlier [8].

2.3. In vitro synthesis of ASB and ASA derivatives

The cDNA coding for residues 39–134 of ASB-N84M was amplified by PCR using a coding primer (AATGCGGCTCCGGACGC-CGGGGCCAGCCGGCCG), that added a *Bsp*EI site 5' to codon 39, and a non-coding primer (GAAGATCTTCTATTTTAGGAG-CTGGGGCAGGA), that added a stop codon followed by a *Bgl*II site 3' to codon 134. The PCR product was cloned as a *Bsp*EI/*Bgl*II fragment into pTD3 [8] in frame with a sequence encoding the signal peptide of preprolactin, thereby substituting the ASA sequences of pTD3 [8]. In vitro expression of the resulting plasmid, designated pRL2, and of the pTD3-derived plasmid pTD17 coding for ASA-F59M, M85T, M87L, M120L (residues 19–200 fused to the signal peptide of preprolactin, ref. [8]) was under control of the SP6-promotor. Both translation products carried a single methionine in their mature sequences (position 59 in ASA or position 84 in ASB).

In vitro synthesis of ASB- and ASA-derived proteins was carried out in a coupled transcription/translation system (TNT, Promega), as described [8]. Rough microsomes from dog pancreas [15] were added at 7.5 equivalents [16] per 50 µl translation mixture. For the co-expression experiment shown in Fig. 4 (lane 3) single translation reactions expressing ASA or ASB, respectively, were mixed prior to incubation at 30°C. This 100 µl sample was split into halves which both were analyzed using either purified ASB-N84M or ASA-F59M protein as a carrier (see below). Purification of translation products imported by the microsomes using differential centrifugation and proteinase K digestion was described earlier [8]. Aliquots (2 µl) were analyzed by SDS-PAGE on high-Tris gels [8] and phosphorimaging (Figs. 1, 3 and 4). The remaining 48 µl (98 µl in the co-expression sample) were used for peptide analysis.

Fig. 1. In vitro modification of ASB in the endoplasmic reticulum. A: A fusion protein consisting of the signal peptide of preprolactin and the N-terminal residues 39–134 of mature ASB-N84M was synthesized in vitro in the presence of [³⁵S]methionine and dog pancreas microsomes. The translation products were analyzed by SDS-PAGE and phosphorimaging. The translation product imported into and processed by the microsomes (mASB) was separated from the non-imported precursor (pASB) by sedimentation of microsomes (lane 1: supernatant; lane 2: pellet). pASB remaining unspecifically bound to the surface of microsomes (upper band in lane 2) was digested by proteinase K and the proteolytic fragments were removed by two further centrifugation steps leading to purified mASB (lane 3). The [³⁵S]methionine is located in the tryptic peptide 3, as is shown in the scheme. B: Radiolabeled mASB (about 20 nCi) was mixed with 40 µg of unlabeled ASB-N84M protein, serving as carrier, and subjected to reductive carboxymethylation, digestion with trypsin and separation of its tryptic peptides by RP-HPLC. In the chromatogram the position of the modified peptide 3 (P3*) is indicated, as identified by mass spectrometry and N-terminal sequencing. The labeled peptide(s) were localized by liquid scintillation counting (see histogram) and identified as derivative(s) of [³⁵S]peptide 3 by radiosequencing (not shown). The radioactive material eluting in the fractions indicated by a horizontal bar, including those containing P3* (indicated in black), was pooled, lyophilized and digested with 0.2 µg endoproteinase AspN in 50 mM ammonium acetate (pH 8.0). C: Upon RP-HPLC of the resulting peptides two radiolabeled peaks were obtained. The left peak (indicated in black) coeluted with P3*C, which was identified by mass spectrometry as indicated. D, E: By radiosequencing the two ³⁵S-labeled peaks (see C) were identified as derivatives of [³⁵S]peptide 3C carrying [³⁵S]methionine 84 in second position. The radioactivity released in each sequencing cycle is given as percentage of total radioactivity recovered in the cleaved amino acids and the non-cleaved material remaining on the sample filter. D: N-terminal sequencing of the left peak (see C) revealed the sequence DMYYT, which corresponds to residues 83–87 of P3*C. The presence of FGly 91 in P3*C (residues 83–95) was verified by mass spectrometry (not shown). E: Sequencing and mass spectrometry of the right peak (see C) did not identify any amino acid or peptide signal. This is in accordance with the presence of unmodified P3C in the in vitro translation product and its absence in the carrier protein. Therefore the sequence DMYYT is given in brackets.

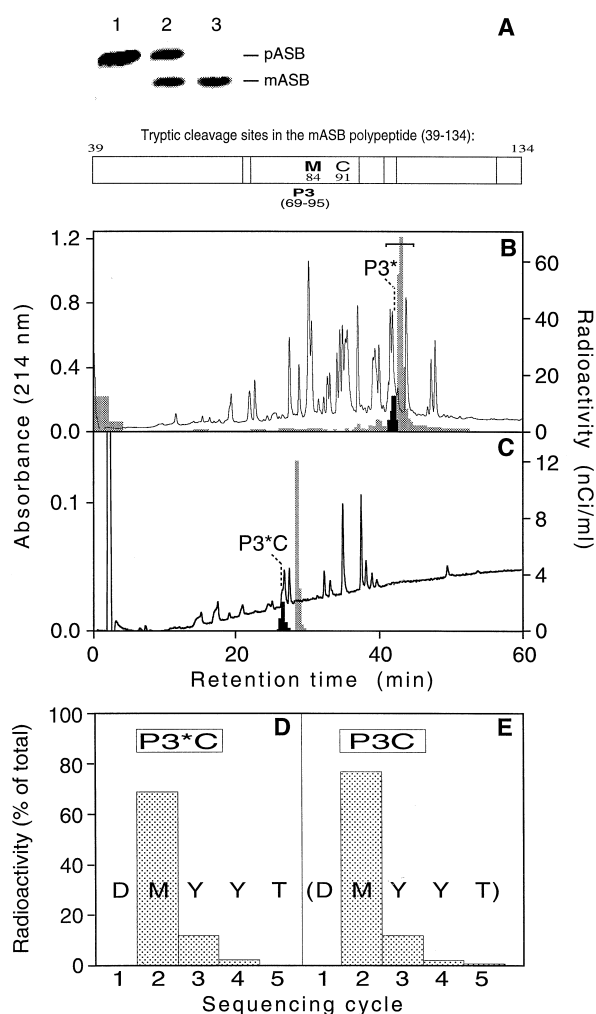
2.4. Peptide analysis

Purified ASB (ASA) in vitro translation/translocation products were mixed with 30–40 µg of unlabeled ASB-N84M (ASA-F59M) carrier protein and subjected to reductive carboxymethylation and generation of peptides by trypsin or endoproteinase AspN, as described [1,8]. Separation of peptides by RP-HPLC, mass spectrometry and sequencing of peptides were also described earlier [1]. The protocols for reaction of peptides with 2,4-dinitrophenylhydrazine and for analysis of the peptide hydrazones are given in ref. [8].

3. Results

3.1. Modification of Cys-91 of ASB in the endoplasmic reticulum

The FGly residue in human ASB is found in position 91 [1]. This position is equivalent to residue 69 of ASA, where a cysteine that is encoded by the ASA gene is incorporated into the primary translation product and becomes converted into FGly upon translocation into microsomes [8]. In order to analyze the presence or absence of FGly-91 in ASB synthesized in vitro in the absence or presence of microsomes, we expressed an N-terminal fragment of ASB-N84M comprising residues 39–134 of ASB-N84M fused to the signal peptide of preprolactin. The exchange of the authentic signal peptide (residues 1–38) for that of preprolactin should ensure efficient translocation into the microsomes. To allow for incorporation of a [³⁵S]methionine-label during translation into the ASB



(39–134) fragment, which lacks methionines, Asn-84 was replaced by methionine. Expression of a full-length ASB-N84M mutant in eukaryotic cells yielded a catalytically active protein (see Section 2) indicating that substitution of Asn-84 by methionine does not interfere with generation of FGly-91. This could also be demonstrated by structural analysis of the ASB-N84M protein (see below).

A cDNA encoding the preprolactin-ASB derivative described above was subjected to coupled *in vitro* transcription and translation in the presence of [35 S]methionine and transport-competent microsomes. The translation product imported into and processed by the microsomes was purified (mASB, Fig. 1A) and mixed with purified ASB-N84M protein serving as carrier. This mixture was subjected to reductive carboxymethylation of cysteines and tryptic digestion. Residue 91 is part of the tryptic peptide 3 of ASB comprising residues 69–95 (Fig. 1A). After separation of the tryptic peptides by RP-HPLC one major 35 S-labeled peak was recovered (Fig. 1B). A part of the radioactivity coeluted with the FGly-91 containing peptide 3 (designated P3*) of the ASB-N84M carrier (Fig. 1B). The latter was identified by mass spectrometry (2886 Da) and amino acid sequencing of the respective fractions. The majority of the radioactivity eluted at a 1.0% higher acetonitrile concentration, where the joined peptides 6 plus 7 (which lack methionines), but no peptide 3 of the ASB-N84M carrier protein could be identified by sequencing and mass spectrometry. Radiosequencing of this material revealed, however, that a methionine was present in position 16, as expected for peptide 3 of the *in vitro* translation product. From experiments with ASA it is known that the peptide 2 carrying the cysteine to be modified elutes at a 1.5% higher acetonitrile concentration than the FGly-containing form of this peptide [1,8]. It was therefore likely that the majority of the radiolabeled peptide of ASB-N84M represented the Cys-

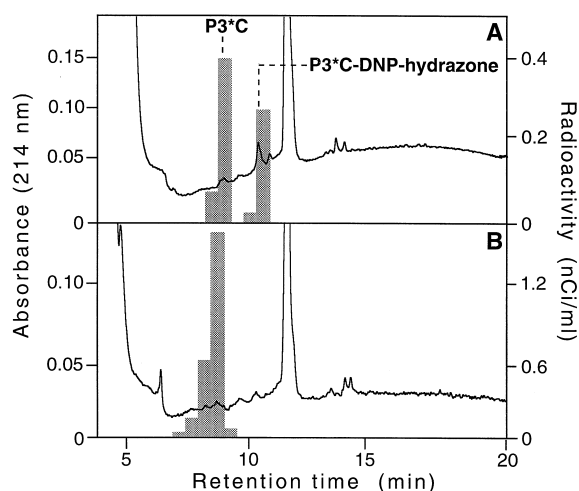


Fig. 2. Presence of an aldehyde group in peptide 3C* after *in vitro* translocation of ASB into microsomes. A: The [35 S]methionine-labeled peptide 3C* coeluting with unlabeled P3* (see Fig. 1C and D) was tested for the presence of an aldehyde group by reaction with DNP-hydrazine. The incubation mixture was subjected to RP-HPLC in order to separate the DNP-hydrazone derivative from the parent peptide and the reagent. The positions of P3* and P3*-DNP-hydrazone are indicated, as identified by mass spectrometry. The radioactivity profile (see histogram) shows that [35 S]P3* was also converted into the hydrazone with an efficiency of approximately 40%. B: Reaction of [35 S]P3C (see Fig. 1C and E) did not give rise to DNP-hydrazone formation.

ASB-C91T92		ASB-T91C92	
-	+	+	
1	2	3	microsomes
			p-ASB
			m-ASB
9	20	6	Modification (P3* as percentage of total P3C)
0	37	0	P3*-DNP-hydrazone (% of total P3* C)

Fig. 3. Conversion of cysteine into FGly in ASB depends on import into microsomes and on the correct position of the cysteine. The *in vitro* translation/translocation product shown in lane 2, representing the ASB construct shown in Fig. 1A, was analyzed for cysteine modification as described in Figs. 1 and 2. The values given for modification represent the percentage of [35 S]P3* C of total [35 S]P3C recovered after HPLC of trypsin- and endoproteinase AspN-generated peptides (see Fig. 1C). In addition, the fraction of [35 S]P3* C that was converted into the corresponding DNP-hydrazone (see Fig. 2) is given. Lane 1 shows the results obtained after analysis of the precursor form of the same ASB construct synthesized in the absence of microsomes. The apparent modification represents background radioactivity coeluting with unlabeled P3* C of the carrier protein, since it did not react with DNP-hydrazine (see text). The same holds true for the translation/translocation product shown in lane 3, which was synthesized in the presence of microsomes but carried the relevant cysteine in position 92 due to an inversion of codons 91 (Cys) and 92 (Thr) in the cDNA.

91 containing form of peptide 3 (designated P3), which is absent in wild-type ASB [1] and in the ASB-N84M carrier (data not shown).

Due to the high hydrophobicity of the large peptide 3 RP-HPLC did not lead to adequate separation of P3* and P3 to allow quantification of modification. Therefore the fractions containing the peak of radioactivity were pooled (see Fig. 1B) and digested with endoproteinase AspN. This generates the 13-mer peptide 3C [1] comprising residues 83–95 of ASB-N84M. By RP-HPLC the 35 S-labeled peptide 3C could be resolved in two forms (Fig. 1C). About 20% of the radioactivity represented [35 S]P3* C, as identified by its coelution with carrier P3* C (mass: 1557 Da) and by radiosequencing (Fig. 1D). About 80% of the radioactivity did not coelute with a carrier peptide, but by radiosequencing could be identified as [35 S]P3C (Fig. 1E).

To examine for the presence of an aldehyde function [35 S]P3C and [35 S]P3* C were subjected to reaction with dimethylphenylhydrazine (DNP-hydrazine) [8]. Only [35 S]P3* C gave rise to hydrazone formation which could be identified after separation from non-reacted [35 S]P3* C by RP-HPLC (Figs. 2 and 3, lane 2). The [35 S]P3* C-DNP-hydrazone coeluted with the unlabeled P3* C-DNP-hydrazone of the carrier, which had the predicted mass of 1737 Da, i.e. 180 Da more than P3* C. It should be noted that hydrazone formation of most FGly-containing peptides is only partial. Quantitative hydrazone formation was found only for smaller and more hydrophilic peptides [8].

Taken together these data demonstrate that about 20% of the ASB-N84M fragment synthesized *in vitro* carried a FGly residue, when translation had been coupled to translocation into microsomal membranes. In the absence of microsomes the translation product representing the precursor form of the preprolactin-ASB-N84M construct carried no aldehyde

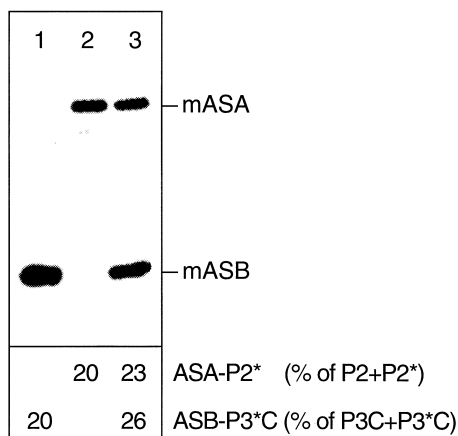


Fig. 4. Simultaneous modification of ASB and ASA. The ASB-N84M construct shown in Fig. 1A (mASB) and the ASA-F59M construct (mASA, see Section 2) were translated and imported into microsomes, each in a 50- μ l reaction mixture (lanes 1 and 2). In addition, duplicates of these translation reactions were mixed prior to incubation at 30°C (lane 3). The phosphorimaging shows 2- μ l aliquots of the import products purified from each translation reaction (i.e. 4% of total in lanes 1 and 2, but only 2% in lane 3) indicating that the two sulfatases are translated and translocated without affecting each other. Note that both mASA and mASB contain a single [35 S]methionine. The purified import products were analyzed for the presence of the FGly-containing peptides P3*C (ASB) or P2* (ASA), respectively. The import products purified from the 100- μ l coexpression sample were analyzed in duplicate. One half was mixed with ASB-N84M carrier protein, the other half with ASA-F59M carrier protein. Analysis of the 35 S-labeled tryptic peptides in a single RP-HPLC run was possible, since the tryptic peptides 2 of ASA and 3 of ASB elute at well separated positions in the acetonitrile gradient. The modification efficiency of ASB was calculated as described in Fig. 3. For ASA the radioactivity coeluting from the RP-column with the tryptic P2* of the carrier ASA-F59M protein and giving a positive reaction with DNP-hydrazine was quantitated (see [8]).

group (pASB; Fig. 3, lane 1). The labeled material coeluting with P3*C of the carrier protein (9% of P3C-associated radioactivity) did not react with DNP-hydrazine and represented contaminating radioactivity. This background was rather high, since the crude translation product present in the reticulocyte lysate had to be analyzed.

The specificity of the modifying machinery was tested by inverting the position of the cysteine to be modified and the position of its C-terminal neighbor (threonine 92). After translation and import of this ASB-mutant into microsomes peptide analysis revealed that only some background radioactivity (6% of P3C-associated radioactivity) coeluted with P3*C of the carrier protein (Fig. 3, lane 3). This radioactive material did not react with DNP-hydrazine.

3.2. Simultaneous modification of ASA and ASB

The relative efficiencies of *in vitro* modification of ASB and ASA, quantitated as percentage of ASB-[35 S]P3*C or ASA-[35 S]P2*, respectively, of total [35 S]P3C or [35 S]P2, respectively, were very similar when assayed in parallel (Fig. 4, lanes 1 and 2). We wanted to know whether the modification efficiency of ASA and/or ASB is affected when the two sulfatases are translated and translocated simultaneously. When co-expressed in the presence of microsomes, ASA and ASB were translated and translocated with the same efficiency, as compared to the single expression controls (Fig. 4, lane 3; it

should be noted that the aliquot subjected to SDS-PAGE in lane 3 is only 50% of that applied in lanes 1 and 2). Analysis of the tryptic peptides of these translocation products, which in the case of ASB had to be digested additionally with endoproteinase AspN (see Fig. 1), clearly showed a similar extent of modification in the co-expressed sulfatases as in the singly expressed ASA or ASB. If changed at all, a slight increase of relative modification was observed for both ASA and ASB (Fig. 4). Calculation of total radioactivity recovered in the HPLC fractions associated with the modified peptides of ASA and ASB revealed that similar amounts of molecules had been modified per equivalent of microsomes [16] in all three samples shown in Fig. 4.

4. Discussion

ASB is subjected to FGly formation in the ER by a mechanism that shares all characteristics observed earlier for ASA [8]. FGly formation depended on protein import into the ER and on the presence of a cysteine in the primary translation product located at the correct position within a sequence that is highly conserved among eukaryotic sulfatases and obviously determines this novel protein modification (see Section 1). This supports the notion that most likely all eukaryotic sulfatases are subjected to this modification by a common modifying machinery located in the ER.

This machinery obviously is saturable under *in vivo* and *in vitro* conditions [1,8]. For ASA it was shown *in vitro* that reducing the expression to about one-twentieth of the level used in the present and in an earlier study [8] doubles the relative modification efficiency from about 20% to about 40% [8]. Using the high expression conditions we observed a similar modification efficiency of about 20% for both ASA and ASB. This efficiency did not drop when ASA and ASB were translocated simultaneously into microsomes. This may indicate that the modifying machinery has a similar affinity for ASA and ASB. However, several other observations are inconsistent with this conclusion. In multiple sulfatase deficiency usually low residual activities of the various sulfatases are detectable. This is attributed to a residual activity of the modifying machinery. Characteristically the residual activity of ASB is the highest among all sulfatases, e.g. 2–4 times higher than that of ASA [17,18]. This suggests that *in vivo* modification of ASB is more efficient than that of other sulfatases. Accordingly, in recombinant ASB Cys-91 is quantitatively modified to FGly, while in recombinant ASA expressed under similar conditions 10–40% of the Cys-69 escape modification [1]. In contrast to these *in vivo* data the *in vitro* data presented in this study would argue for a similar affinity of the modifying machinery for ASB and ASA. The similar and overall rather low modification efficiencies observed in the *in vitro* system, however, may in fact result from limitation at a certain step of modification that is not limiting *in vivo*. If the translocation and modifying machineries are coupled at the luminal side of the ER membrane, limited modification may merely reflect an excess of translocation over modification under *in vitro* conditions. Until now attempts to uncouple translocation and modification, e.g. by studying modification of a sulfatase precursor polypeptide in a microsomal detergent extract, have proven unsuccessful (not shown). Obviously, the translocation and modification machineries have to be co-constituted in order to develop our *in vitro* system into an

assay that is suitable to biochemically identify the modifying enzyme(s).

Acknowledgements: We thank Meike Pauly-Evers and Christoph Peters (Freiburg) for providing the affinity column for ASB purification. The technical assistance of Petra Schlotterhose is gratefully acknowledged. This work was supported by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

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