Characterization of Posttranslational Formylglycine Formation by Luminal Components of the Endoplasmic Reticulum*

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C FORMYLGLYCINE is the key catalytic residue in the active site of sulfatases. In eukaryotes formylglycine is generated during or immediately after sulfatase translocation into the endoplasmic reticulum by oxidation of a specific cysteine residue. We established an in vitro assay that allowed us to measure formylglycine modification independent of protein translocation. The modifying enzyme was recovered in a microsomal detergent extract. As a substrate we used ribosome-associated nascent chain complexes comprising in vitro synthesized sulfatase fragments that were released from the ribosomes by puromycin. Formylglycine modification was highly efficient and did not require a signal sequence in the substrate polypeptide. Ribosome association helped to maintain the modification competence of nascent chains but only after their release efficient modification occurred. The modifying machinery consists of soluble components of the endoplasmic reticulum lumen, as shown by differential extraction of microsomes. The in vitro assay can be performed under kinetically controlled conditions. The activation energy for formylglycine formation is 61 kJ/mol, and the pH optimum is ~10. The activity is sensitive to the SI/SS equilibrium and is stimulated by Ca2+. Formylglycine formation is efficiently inhibited by a synthetic sulfatase peptide representing the sequence directing formylglycine modification. The established assay system should make possible the biochemical identification of the modifying enzyme.

Pro- and eukaryotic sulfatases form a protein family whose members have similar structure and function (1–4). Furthermore, they share a unique protein modification (5–9). At their active sites, sulfatases carry a C FORMYLGLYCINE (FGly)1 residue that is essential for catalytic activity. Structural and enzymatic studies of mutants of arylsulfatase A (ASA), in which the FGly was replaced by a serine or alanine, and, namely, the recent studies of mutants of arylsulfatase A (ASA), in which the FGly side chain is present as an aldehyde hydrate in the resting state of the enzyme (4, 10–14). During catalysis one of the two hydroxyls of this geminal diol performs a nucleophilic attack on the sulfur of the substrate’s sulfate group leading to a covalently sulfated enzyme intermediate (4, 11, 12). The second hydroxyl is required for desulfation of the intermediate and for concomitant aldehyde regeneration. Accordingly, the latter (desulfation) but not the former (sulfation) step is blocked in the serine mutant mentioned above, which lacks the second hydroxyl (11). The key function of the FGly residue in this reaction mechanism explains the critical role of the posttranslational generation of this residue in the biosynthesis of enzymatically active sulfatases. In humans the genetic defect of this posttranslational modification is the molecular cause of multiple sulfatase deficiency, a rare but fatal metabolic disorder leading to synthesis of catalytically inactive sulfatase polypeptides (5, 15).

In bacteria, FGly can be generated by two systems, a cysteine-FGly-converting system present in Pseudomonas aeruginosa and Escherichia coli (8) and a serine-FGly converting system present in Klebsiella pneumoniae (7) and E. coli (3, 9). The two systems obviously show no functional overlap (8). Only the serine but not the cysteine system involves an iron–sulfur protein, termed AtsB in K. pneumoniae (9). It must still be clarified whether AtsB represents the modifying enzyme or merely a specific cofactor.

In eukaryotes, the modifying machinery is also unknown. FGly is generated by oxidation of a conserved cysteine residue encoded by the sulfatase genes. During synthesis of sulfatases this cysteine is incorporated into the nascent polypeptide (16). It is converted to FGly 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 (16, 17). The FGly modification is directed by an autonomous linear sequence motif (CXPSRXXX(L/M)/TG(I/K/V/L)) that is located within the N-terminal 80–120 amino acid residues of the sulfatases, i.e. 60–100 residues downstream of the signal peptide cleavage site (18). Using ribosome-associated nascent chain complexes (RNCs) we could show that, in an arrested translocation intermediate, the cysteine 69 of ASA was accessible inside the microsomes, as evidenced by N-glycosylation of an engineered glycosylation site at position 87; however, FGly formation (and also signal peptide cleavage) was observed only after release of the nascent chain from the ribosome by puromycin (16). Thus, FGly modification occurs after or at a late stage of protein translocation. Short sulfatase sequences (16 residues) comprising the modification motif were sufficient for FGly generation even when inserted into a heterologous polypeptide background, indicating that the modification does not depend on folding of sulfatases but rather occurs as long as the newly

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1 The abbreviations used are: FGly, C FORMYLGLYCINE; ASA, arylsulfatase A (cerebrosidase-3-sulfate 3-sulfohydrolase, EC 3.1.6.8); DNPH, dinitrophenylhydrazine; DNP-hydrazone, dinitrophenylhydrazone; ER, endoplasmic reticulum; GSH, reduced glutathione; GSSG, oxidized glutathione; RNCs, ribosome-associated nascent chain complexes; RP-HPLC, reversed phase-high performance liquid chromatography; CAPS, 3-cyclohexylamino)propanesulfonic acid.

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synthesized polypeptide is largely unfolded (16–18).

Until now FGly modification could only be studied in intact microsomal membranes, i.e. coupled to cotranslational protein translocation. Investigating FGly formation independent of translocation and, if possible, independent of intact ER membranes would be the first step toward a biochemical description of the enzymatic machinery involved and eventually could allow to identify this machinery after chromatographic purification. Here we describe the development of such an assay system and its application to elucidate basic aspects of FGly modification.

EXPERIMENTAL PROCEDURES

Extraction of Microsomal Membranes—Dog pancreas (19) and cow pancreas microsomes (20) were prepared as described previously. For definition of microsomes see Ref. 21. The integrity of microsomes as well as the efficiency of extraction/solubilization by detergent (see below) was routinely checked by Western blotting using anti-protein disulfide isomerase (StressGen), anti-calreticulin, and anti-Sec61α antibodies. The latter two antibodies were kind gifts of Phuc Nguyen Van (Göttingen) and Enno Hartmann (Göttingen), respectively.

For total solubilization, dog pancreas microsomes were incubated at 1.0–2.0 mg/ml (6) in extraction buffer (see Table I). A total of 12.5 mM dithiothreitol was added. Reduction of disulfide bridges was performed on a Fast Desalting PC3.2/10 column using the SMART system (Amersham Biosciences, Inc.). The incubation was stopped by adding 167 µl of preheated (80 °C) carboxymethylation buffer (400 mM Tris-HCl (pH 8.6), 10 mM EDTA, 6 mM guanidine hydrochloride) and heating for 10 min at 80 °C.

The extraction of dog pancreas microsomes (Table I) resulted in membrane association of RNCs and, after addition of detergent, was used as a blank for the detection of FGly modification activity (see also Fig. 4).

For differential extraction, dog pancreas microsomes were incubated at 1.3 eq/ml in extraction buffer (50 mM HEPES-KOH (pH 7.5), 1 mM magnesium acetate, 125 mM sucrose, 150 mM potassium acetate) that had been supplemented with 0–1 mM deoxy Big Chap (see Fig. 4). After 30 min on ice, the extract was recovered from the supernatant of a 30-min centrifugation at 200,000 × g in a Beckman TLA100.3 rotor.

The incubation of dog pancreas microsomes (see also Fig. 4) was performed on a Fast Desalting PC3.2/10 column using the SMART system (Amersham Biosciences, Inc.). The labeled peptides and their derivatives were quantitated by liquid scintillation counting. Modification efficiencies were calculated as described in Ref. 18, and the amount of modified peptide is given as the percentage of total, i.e. modified plus unmodified peptide.

RESULTS

FGly Modification Assay—In the standard assay (see Figs. 5 and 6, Table I) 30 eq of reticuloplasm (or gel-filtered reticuloplasm) was diluted to 87.5 µl with extraction buffer (no detergent) and incubated with 5 µl of salt-washed RNCs (pMB2/AluNI-programmed) and 7.5 µl of puromycin (final concentration 1.5 mM) for 20 min at 37 °C. Gel filtration of the reticuloplasm was performed on a Fast Desalting PC3.2/10 column using the SMART system (Amersham Biosciences, Inc.). The incubation was stopped by adding 167 µl of preheated (80 °C) carboxymethylation buffer (400 mM Tris-HCl (pH 8.6), 10 mM EDTA, 6 mM guanidine hydrochloride) and heating for 10 min at 80 °C.

Aliquots of the assay samples were routinely checked for recovery and, where conceivable, signal peptide cleavage of the substrate polypeptide by SDS-PAGE (23) and phosphorimaging (BAS 1000, Raytest). Further aliquots were analyzed by Western blotting for recovery of membrane and/or luminal ER proteins (see above).

Carboxymethylation and Peptide Analysis—The stopped assay mixture was supplemented with 15–30 µg of unlabelled ASA-F59M carrier protein, which served as an internal standard for peptide analysis (16, 18), and was concentrated to 167 µl by a Speed-Vac concentrator. Reductive carboxymethylation and generation of tryptic peptides were carried out as described previously (16), using 12.5 mM dithiothreitol for reduction, 36 mM iodoacetic acid (Sigma-I-8136) for carboxymethylation and 3% (w/v) of modified trypsin (sequencing grade, Roche Molecular Biochemicals) for tryptic digestion. Separation of tryptic peptides by RP-HPLC, mass spectrometry, and sequencing of unlabeled peptides (from carrier ASA protein) also were described earlier (5).

Michaelone-labeled peptides were identified by radiosequencing (5) and, in control experiments using pTD17/AluNI-programmed RNCs (16), by their coelution with the corresponding unlabeled peptides coming from the carrier protein (not shown, cf. Refs. 16, 18). To assay for the presence of an aldehyde group, purified 35S-peptide was subjected to reaction with dinitrophenylhydrazine (DNPH) (16). Unreacted 35S-peptide and its dinitrophenylhydrazone (DNPH-hydrazone) derivatives were separated by RP-HPLC on a C18 reversed-phase column (Amersham Biosciences, Inc.). The labeled peptides and their derivatives were quantitated by liquid scintillation counting. Modification efficiencies were calculated as described in Ref. 18, and the amount of modified peptide is given as the percentage of total, i.e. modified plus unmodified peptide.
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FIG. 1. The two substrate polypeptides used for in vitro FGly modification. The two translation products shown were generated by in vitro transcription and translation of plasmids pTD31 and pMB2, which had been cleaved by Alu/Sal in the coding region 3’ of ASA codon 158 (for details see “Experimental Procedures”). Polypeptide I consists of the preprolactin signal peptide, a TPDM linker peptide (italics), and ASA residues 65–158, as indicated. After signal peptide cleavage and tryptic digestion, the tryptic peptide 1 (P1) consists of 13 amino acid residues comprising the only methionine of the mature polypeptide (used for 35S labeling) and the cysteine 69 of ASA to undergo FGly residues comprising the only methionine of the mature polypeptide tryptic digestion, the tryptic peptide 1 (P1) carried the only methionine and cysteine 69, as indicated. The two translation products were characterized with respect to size, signal peptide cleavage (see Fig. 2), and ribosome association via their C-terminal tRNA (see Fig. 3) using SDS-PAGE and phosphorimaging. The position of the labeled methionines was verified by radiosequencing of both intact translation products and their tryptic peptides. Basic FGly modification experiments performed with polypeptide I were controlled by using a related polypeptide comprising residues 19–158 of ASA-F59M (16). In these controls the relevant tryptic peptide generated from the in vitro translation product was identical to the tryptic peptide generated from added ASA-F59M carrier protein (see “Experimental Procedures”), as evidenced by coelution of these peptides during RP-HPLC and by DNP-hydrazone formation. Note that throughout this study numbering of amino acid residues refers to the precursor, i.e. signal peptide containing form of authentic ASA.

signal peptidase-cleaved, ASA fragments (Fig. 2A). This was shown by reductive carboxymethylation and tryptic digestion of the incubation mixture followed by RP-HPLC analysis of tryptic peptides that allows for separation of the modified and non-modified forms of peptide 1 (ASA residues 65–73 preceded by the tetrapeptide TPDM) containing FGly or carboxymethylcysteine, respectively, at position 69 (Fig. 2A). Radiosequencing identified both labeled peptides as variant forms of the tryptic peptide 1 (not shown). The presence of FGly in the early eluting form (27 min) but not in the later eluting form (29 min) of peptide 1 was verified by reaction with dinitrophenylhydrazine (DNPH). Only the former could be converted into a hydrazide derivative (72% efficiency, not shown). Hydrazine formation depends on the presence of an aldehyde group and increases the hydrophobicity, which is utilized to separate peptide 1 from its hydrazide derivative (not shown, see Refs. 16–18).

When RNCs were incubated (20 min, 37 °C) with a detergent extract (2.5% deoxy Big Chap, 400 mM potassium acetate) of dog pancreas microsomes (20 eq) in the presence of puromycin, we also observed partial signal peptide cleavage (35%, not shown). FGly analysis showed that the mature form was efficiently modified, as revealed by RP-HPLC of tryptic peptides. 80% of the signal peptidase-cleaved ASA fragments contained FGly (Fig. 2B), and 90% thereof could be converted to a DNP-hydrazone (not shown). Thus, modification by a detergent extract was more efficient than by intact microsomal membranes. Analysis of the precursor form of the substrate, containing the uncleaved signal peptide (65% of total molecules), was difficult due to the large size and high hydrophobicity of the corresponding tryptic peptide. After cleavage of this peptide with endoproteinase Asp-N, however, we found also that the precursor form was at least partially modified (not shown).

**FGly Modification Does Not Depend on the Presence of a Signal Peptide**—Apart from its function in targeting and translocation of the sulfatase polypeptide, the signal peptide may assist in maintaining an unfolded, modification competent state of the sulfatases’ N-terminal region where the cysteine to be modified is located in all known sulfatases (see the introduction). To test for a possible influence of the signal peptide on FGly formation, we eliminated the signal sequence and substituted it by the sequence MGLRMPD (italics) and the extract supernatant (35% signal peptide cleavage) and half from the pellet (no signal peptide cleavage), as checked by SDS-PAGE and phosphorimaging (not shown). After reductive carboxymethylation and tryptic digestion, the FGly-modified and non-modified version of the 35S-labeled peptide 1 (P1* and P1; cf. Fig. 1) of signal peptidase-cleaved polypeptides were separated by RP-HPLC, as shown for the pellet of intact microsomes (A) and the extract supernatant (B). Quantification of the peptides by liquid scintillation counting (shaded area) revealed that 22% (A) and 80% (B) of peptide 1 carried the FGly modification. For analysis of signal peptide-containing peptide 1 see text. The unlabelled tryptic peptides shown in the RP-HPLC chromatograms (UV absorbance) derived from proteins in the assay mixture and from purified ASA carrier protein, which was added as an internal standard prior to carboxymethylation (see “Experimental Procedures”).

**FIG. 2. FGly modification by a microsomal detergent extract.** Salt-washed RNCs containing ASA polypeptides fused to the signal sequence of preprolactin (Fig. 1, substrate I) were mixed with 20 eq of intact dog pancreas microsomes (A) or a fully solubilized detergent extract thereof (B). After 5 min on ice, the assay mixture (25 µl) was supplemented with puromycin (1.5 mM) and incubated for 20 min at 37 °C. The translocation/ modification assay samples were chilled on ice and subjected to centrifugation (30 min, 2 °C, 200,000 × g). In A, 95% of the polypeptides sedimented with the microsomes, 44% of which were cleaved by signal peptidase, whereas in B about half of the polypeptides were recovered from the supernatant (35% signal peptide cleavage) and half from the pellet (no signal peptide cleavage), as checked by SDS-PAGE and phosphorimaging (not shown). After reductive carboxymethylation and tryptic digestion, the FGly-modified and non-modified versions of the 35S-labeled peptide 1 (P1* and P1; cf. Fig. 1) of signal peptidase-cleaved polypeptides were separated by RP-HPLC, as shown for the pellet of intact microsomes (A) and the extract supernatant (B). Quantification of the peptides by liquid scintillation counting (shaded area) revealed that 22% (A) and 80% (B) of peptide 1 carried the FGly modification. For analysis of signal peptide-containing peptide 1 see text. The unlabelled tryptic peptides shown in the RP-HPLC chromatograms (UV absorbance) derived from proteins in the assay mixture and from purified ASA carrier protein, which was added as an internal standard prior to carboxymethylation (see “Experimental Procedures”).
introduced a glycine in position 2 to ensure methionine removal. It turned out that the release was very efficient (100 and 92%) in the two samples that had received puromycin prior to or simultaneously with the addition of the microsomal extract. The FGly modification efficiency observed under "concomitantly released" conditions (37%, i.e. 4.6% per eq) was twice as high as under "pre-released" conditions (19%) (Fig. 3). In the puromycin-free sample, only 55% of total polypeptides sedimented with the ribosomes, suggesting that a significant portion of the RNCs were disassembled during incubation at 37 °C or later. Only 10% of the ribosome-associated polypeptides carried the FGly modification, whereas the degree of modification was 28% for the released polypeptides coming from disassembled RNCs (Fig. 3). In conclusion, releasing the polypeptide from the ribosome strongly improves the FGly modification efficiency. On the other hand, ribosome association helps to maintain the modification competence, because the modification of pre-released polypeptides was clearly less efficient than modification of concomitantly released polypeptides.

Localization of the Modifying Activity in the ER Lumen—Using the in vitro assay ("concomitantly released" conditions) we determined whether the components of the FGly-generating machinery are part of the ER membrane or of the luminal content or both. Microsomes were extracted at 150 mM potassium acetate with increasing concentrations of detergent and separated into a supernatant and pellet fraction by centrifugation. Increasing concentrations of detergent solubilized increasing amounts of the FGly-modifying activity (Fig. 4A). At 1 mM concentration of deoxy Big Chap, 90–100% of this activity was recovered in the supernatant and virtually no activity remained in the membrane pellet. Western blot analysis of the supernatant and pellet fractions revealed that luminal components like calreticulin were extracted efficiently at 1 mM deoxy Big Chap, whereas membrane proteins like Sec61α essentially all stayed in the membrane pellet (Fig. 4B). Thus, the modifying machinery is part of the soluble components of the ER lumen referred to as reticuloplasm.

Reconstitution experiments led to the same conclusion. After removing the detergent from a fully solubilized microsomal extract by dialysis, the reconstituted membranes were separated from non-reconstituted soluble proteins by ultracentrifugation. Both fractions, before being tested for FGly-modifying activity, were again treated with solubilizing concentrations of detergent. It turned out that the FGly-forming activity was solely associated with the soluble and not with the membrane fraction (data not shown).

Kinetic and Biochemical Characterization of FGly Modification—For a reliable analysis of FGly modification in dependence of various parameters, we had to define standard assay conditions providing a linear dependence of FGly generation on incubation time and amount of modifying enzyme. Reticuloplasm was prepared as described above using bovine pancreas microsomes. Their specific modification activity was 2–4-fold lower as compared with canine pancreas microsomes. Aliquots of reticuloplasm corresponding to 45 eq of microsomal membranes were incubated in a final volume of 100 μl with salt-washed RNCs (~30,000 dpm, estimated to correspond to ~15 fmol of polypeptide substrate) for 0–60 min at 37 °C and then stopped by addition of preheated (80 °C) carboxymethylation buffer containing 6 M guanidine hydrochloride. Under the indicated conditions FGly formation is linear with time for 20 min (Fig. 5A) but levels off at longer incubation times, when

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**Fig. 3. Influence of ribosome association on FGly modification of ASA polypeptides.** ASA polypeptides lacking a signal peptide (Fig. 1, substrate III) were incubated for 20 min at 37 °C with a fully solubilized microsomal detergent extract (cf. Fig. 2B) under three different conditions, as indicated. In two samples the substrate polypeptide was added in the form of salt-washed RNCs, from which the nascent chains were concomitantly released or not released due to the simultaneous addition of puromycin (1.5 mM) or cycloheximide (2 mM), respectively. In a third sample, the ASA substrate was added as pre-released polypeptides, i.e. after puromycin treatment of RNCs and removal of ribosomes by sedimentation. Following incubation, all three samples were chilled on ice and subjected to centrifugation (30 min, 2 °C, 200,000 × g) to separate ribosome-associated from non-associated polypeptides, which were then subjected to FGly determination. 92 and 37 and 19%, respectively, carried an FGly modification. In the sample, 55% of polypeptides (with 10% FGly modification) were recovered in the supernatant of the concomitantly released conditions for the released polypeptides coming from disassembled RNCs (Fig. 3). In conclusion, releasing the polypeptide from the ribosome strongly improves the FGly modification efficiency. On the other hand, ribosome association helps to maintain the modification competence, because the modification of pre-released polypeptides was clearly less efficient than modification of concomitantly released polypeptides.

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**Posttranslational Formylglycine Formation in the ER Lumen**

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**Table 1. Influence of Ribosome Association on the Modification of Sulfatase Polypeptides**

| Condition          | Polypeptides | FGly |
|--------------------|--------------|------|
| Concomitantly released | 92%          | 37%  |
| Non-released        | 45%          | 28%  |
| Pre-released        | 10%          | 19%  |

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**Legend:**

- **salt-washed RNCs**
  - + microsomal extract
  - + puromycin
  - Removal of ribosomes

**Diagram with Legend:**

- **Poly-peptides:**
  - supernatant: 92%
  - supernatant: 45%
  - pellet: 10%
  - supernatant: 19%

**Figure 3:**

- **Concomitantly released**
  - 37°C
  - + puromycin

- **Non-released**
  - 37°C
  - + cyclo-heximide

- **Pre-released**
  - 37°C
  - + cyclo-heximide

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**Posttranslational Formylglycine Formation in the ER Lumen**

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**References:**

- See Ref. 24.
- For the alteration of the modifying activity.
- For the localization of the modifying activity.
- For the kinetic and biochemical characterization of FGly modification.

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within the range expected for an enzyme-mediated reaction.

The synthetic ASA 16-mer peptide PVS(LCTPSRAALLTG), which comprises all structural information to direct FGly modification in ASA-preprolactin fusion proteins (see the introduction), has been shown to inhibit FGly formation by intact microsomal membranes (18). Varying the peptide concentration from 0 to 3 μM we observed half-maximum inhibition (IC50) of FGly formation by the reticuloplasm at 134 nM (Fig. 5D). This concentration is about 100-fold lower than the IC50 of ~20 μM observed with intact microsomes. On the other hand, 134 mM is about 1000-fold higher than the concentration of the radioactive substrate polypeptide in our assay (~0.15 mM).

FGly formation showed a rather unusual pH dependence. The pH was adjusted by appropriate HEPES/Tris or HEPES/CAPS buffers that were added to the assay mixture immediately before starting the modification reaction (see Fig. 5E). It turned out that FGly modification was fastest at pH 10.0–10.5 with about 50% activity at pH 7.0 and 30% at pH 11.9. Thus, FGly formation can be assayed at a broad pH range (pH 7–11).

The FGly-forming enzyme is a component of the ER lumen where an oxidizing environment is maintained by ~1 mM glutathione (reduced (GSH) plus oxidized (GSSG) glutathione) with a GSH/GSSG ratio of 1:1 to 3:1 (25). To test for a possible influence of glutathione, we investigated FGly modification using reticuloplasm from which all low molecular weight components were removed by gel filtration (see “Experimental Procedures”). Upon addition of glutathione to our assay we observed clear stimulation by 1 mM GSH and clear inhibition by 1 mM GSSG (Table I). The two effects compensated each other when using mixtures of GSH and GSSG. Actually, it turned out that reducing conditions are required, with 2.5 mM GSH or 2.5 mM dithiothreitol giving optimal results (Table I). Surprisingly, 10 mM GSH, as present in the cytoplasm of eukaryotic cells (25), strongly inhibited FGly formation.

The ER has a much higher Ca2+ concentration than the cytosol and many Ca2+-binding proteins are found in the ER lumen. To investigate the influence of Ca2+ ions on FGly formation, again gel-filtered reticuloplasm was used. As shown in Fig. 6, Ca2+ clearly stimulated the modification reaction at low micromolar concentrations with a saturation at ~15 μM.

**DISCUSSION**

The aim of this study was to establish an *in vitro* assay system that makes possible the measurement of FGly formation independent of protein translation and translocation by using microsomal detergent extracts as a source for the FGly-generating activity. Such an assay system is the basis for a biochemical characterization and, eventually, identification of the modifying enzyme.

Toward this goal we developed a suitable [35S]methionine-labeled sulfatase polypeptide substrate that was translated *in vitro* by an mRNA-programmed reticulocyte lysate. To uncouple FGly modification from translation we used RNC technology that allowed us to isolate nascent sulfatase polypeptides from the translation system by sedimentation of ribosomes through a high salt sucrose cushion. The polypeptides of these salt-washed RNCs could be successfully targeted to and translocated by intact microsomes, as evidenced by signal peptide cleavage and FGly modification after addition of puromycin (Fig. 2A). FGly modification also was observed when microsomal membranes, carrying targeted RNCs, were treated with detergent prior to the puromycin chase (not shown). These findings showed that FGly formation may occur independent of translation and does not strictly require an intact microsomal membrane.

To further exclude a possible role of the translocon we had to uncouple FGly modification from both translation and translo-
cation. This was achieved when incubating salt-washed RNCs with a fully solubilized microsomal detergent extract. Upon addition of puromycin, highly efficient FGly modification was observed (Fig. 2B). In fact the modification efficiency was about 4-fold higher than that observed in a coupled translocation/modification assay using intact microsomes. This indicates that a process upstream of FGly modification (e.g. protein translocation) is the limiting step in the in vitro system using intact microsomes.

In intact microsomes we had been unable to temporally...
different concentrations (0
ification activity under standard conditions and in the presence of
or dithiothreitol at the indicated concentrations. The results of two
independent experiments (A and B) are shown.

| GSH | GSSG | Dithiothreitol | FGly-modified peptide |
|-----|------|---------------|----------------------|
| A   |      |               |                      |
| 0   | 0    | 6.1           |                      |
| 1.0 | 0    | 11.6          |                      |
| 0.5 | 0.5  | 7.0           |                      |
| 0   | 1.0  | 2.1           |                      |
| 0.25| 0    | 10.0          |                      |
| 2.5 | 0    | 14.8          |                      |
| 10  | 0    | 0.4           |                      |
| B   |      |               |                      |
| 0   | 0    | 9.4           |                      |
| 2.5 | 0    | 17.7          |                      |
| 2.083| 0.417| 11.7         |                      |
| 1.875| 0.625| 11.4        |                      |
| 1.667| 0.533| 8.8         |                      |
| 1.25| 1.25 | 4.6           |                      |
|     | 1.25 | 14.9          |                      |
|     | 2.5  | 18.6          |                      |

TABLE I

Cow pancreas reticuloplasm was passed over a gel filtration column (see “Experimental Procedures”) and assayed for FGly modification activity under standard conditions and in the presence of GSH, GSSG, or dithiothreitol at the indicated concentrations. The results of two independent experiments (A and B) are shown.

FIG. 6

Stimulation of FGly formation by calcium ions. Cow pancreas reticuloplasm was passed over a gel filtration column (Sephadex G-25, see “Experimental Procedures”) and assayed for FGly modification activity under standard conditions and in the presence of different concentrations (0–15 μM) of CaCl₂, as indicated. At higher CaCl₂ concentrations (up to 100 μM) similar modification efficiencies were observed as at 15 μM CaCl₂ (not shown).

separate FGly formation from signal peptide cleavage (16). However, using a detergent extract as an enzyme source, we found that FGly modification was independent of the presence of a signal sequence at the substrate’s N terminus. Thus, the signal peptide, at least when microsomal membranes are solubilized, is not involved in maintaining a modification competent state of the adjacent mature polypeptide sequence. The cysteine to be modified in the employed substrate polypeptide is located only nine residues C-terminal of the signal peptide cleavage site. This finding does not rule out that the signal peptide plays a role in vivo by anchoring the N terminus in the membrane.

The attachment of the sulfatase nascent chain to the ribo-

some via the C-terminal tRNA (non-released conditions, Fig. 3) largely impaired FGly formation, because the modification was mainly found in the ribosome-released polypeptides. Thus, non-released chains obviously are difficult to modify, albeit the cysteine 69, which has a distance of 89 residues to the C terminus, is accessible outside the ribosome (see the introduction and Ref. 16). This does not necessarily mean that FGly formation only occurs posttranslationally, i.e. after completion of translation/translocation, because we analyzed a translation intermediate artificially arrested at the ribosome after synthesis of only 30% of the entire sulfatase protein. On the other hand, FGly formation indeed occurred under strictly posttranslational conditions, when the detergent extract was incubated with ribosome-free substrate polypeptides, i.e. after their release from the ribosomes by puromycin and removal of ribosomes (Fig. 3). However, the modification efficiency was doubled, when the substrate polypeptides were released concomitant to the addition of the microsomal extract. Thus, FGly formation obviously is optimal immediately after releasing the nascent polypeptide from the ribosome, as may be the case in vivo at a late cotranslational or early posttranslational step of protein translocation. Hence, ribosome/translocon association may assist in maintaining a modification competent state of the sulfatase polypeptide.

The FGly-modifying machinery consists of soluble compon-

ent(s) of the ER lumen, as could be shown by differential extraction of microsomes with low concentrations of detergent. Under conditions that extract the luminal proteins but leave the membrane proteins in the membrane fraction, almost all FGly-forming activity was recovered in the soluble fraction. Accordingly, after reconstitution of membranes by detergent removal (dialysis) from a fully solubilized detergent extract, the FGly-generating activity was not associated with the membranes (proteoliposomes) but with the non-reconstituted soluble proteins.

Kinetic characterization of the FGly-forming activity in the reticuloplasm revealed typical enzymatic properties with a characteristic temperature dependence (activation energy 61 kJ/mol) and strong inhibition by a synthetic peptide comprising the modification motif (IC₅₀ 135 nM). This IC₅₀ value may be taken as a figure of the enzyme’s substrate affinity constant. A true Kₘ value could not be determined by saturation studies, because we had to use in vitro synthesized substrate polypeptides (~0.15 nM). At this low substrate concentration pre-
steady-state conditions may prevail with binding (and not catalysis) being the rate-limiting step. The slow kinetics observed (1% turnover in 20 min per equivalent of cow pancreas microsomes) may reflect this limitation or, alternatively, may indicate a very low abundance of the modifying machinery in the reticuloplasm.

This machinery shows some characteristics that are not un-
expected for an enzyme residing in the lumen of the ER. It is stimulated by calcium ions (Fig. 6) and is sensitive to the SH/SS-redox milieu (Table I). High concentrations of GSH but also moderate concentrations of GSSG inhibited FGly formation. Optimum conditions were observed at 2.5 mM GSH or dithiothreitol indicating that these thiols are required to pre-
vent oxidation of the enzyme’s cysteines. The substrate’s cysteine obviously was not affected in these experiments, because only pretreatment of reticuloplasm with dithiothreitol led to stimulation of FGly modification activity, whereas pretreat-
ment of RNCs with dithiothreitol had no or a partial inhibitory effect (not shown). Passing the reticuloplasm through a gel filtration column did not reduce its specific modifying activity (in the presence of dithiothreitol and Ca²⁺), suggesting that no diffusible low molecular weight cofactor is required for in vitro
FGly formation. More specifically, a possible role of GSSG as a cofactor that is directly involved in cysteine oxidation is highly unlikely. However, we cannot definitely exclude that GSH/GSSG is required to maintain a redox potential favoring cysteine oxidation.

The pH dependence of modification, however, was rather surprising. Its high alkaline pH optimum (10.0–10.5) is difficult to explain. It may reflect that OH− ions play a role, as could be envisaged if FGly formation occurs in two steps, namely an enzyme-mediated oxidation of the cysteine’s thiol to a thioaldehyde group (–CH=S) that subsequently is spontaneously hydrolyzed to eliminate H2S and yield FGly (see also Refs. 5 and 8). The hydrolytic step may be stimulated by alkaline pH. Thus, the broad pH profile observed (Fig. 5E) may represent a composite pH optimum curve of the oxidation and the hydrolytic step, the latter being rate limiting at low pH and the former being rate limiting at high pH. To test for this hypothesis, we chilled a 20 min/37 °C modification reaction on ice, shifted its pH from 7.5 to 10.5, and prolonged the incubation for another 20 min at 0 °C. This treatment, however, did not increase the modification efficiency as compared with non-shifted or non-prolonged control samples (data not shown).

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Characterization of Posttranslational Formylglycine Formation by Luminal Components of the Endoplasmic Reticulum
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