Membrane-bound Cytochrome b_5 Reductase (Methemoglobin Reductase) in Human Erythrocytes

STUDY IN NORMAL AND METHEMOGLOBINEMIC SUBJECTS

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ABSTRACT In this study we present evidence that in human erythrocytes NADH-cytochrome b_5 reductase (methemoglobin reductase) is not only soluble but also tightly bound to the membrane. The membrane methemoglobin reductase-like activity is unmasked by Triton X-100 treatment, and represents about half of the total activity in the erythrocytes. Like the amphiphilic microsomal-bound cytochrome b_5 reductase, the erythrocyte membrane-bound enzyme is solubilized by cathepsin D. Because this treatment is effective on unsealed ghosts but not on resealed (inside-in) ghosts, it is concluded that the enzyme is strongly bound to the inner face of the membrane. The erythrocyte membrane enzyme is antigenically similar to the soluble enzyme. The two forms of enzyme are specified by the same gene, in that both were found defective in six patients with recessive congenital methemoglobinemia. We suggest that the cytochrome b_5 reductase of the erythrocyte membrane is the primary gene product. A posttranslational partial proteolysis probably gives rise to the soluble form of the enzyme, which serves as a methemoglobin reductase.

INTRODUCTION

The so-called methemoglobin-reductase or NADH-diaphorase has been described long ago as a soluble erythrocyte enzyme that has a major role in the enzymatic reduction of methemoglobin (1). An inherited homozygous defect of this enzyme produces congenital recessive methemoglobinemia (2). The enzyme locus (DIA_1) has been assigned to chromosome 22 (3, 4). It has been demonstrated that this erythrocyte enzyme is actually a soluble NADH-cytochrome b_5 reductase (5–8) that reduces a soluble cytochrome b_5. The reduced cytochrome b_5 interacts directly with methemoglobin (5, 9). In the other cells, the NADH-cytochrome b_5 reductase is predominantly bound to the membranes: endoplasmic reticulum (10) and mitochondria (11). However, a soluble form of the enzyme has also been found in the cytosolic fraction of human placenta (8) and rabbit liver (12).

In this paper, we report the finding of a NADH-cytochrome b_5 reductase that is strongly attached to the inner face of the erythrocyte membrane and is released either by detergent treatment or by partial digestion by cathepsin D. The immunologic and genetic characterization of the membrane-bound enzyme suggests that it is produced by the same gene (DIA_1) as the soluble erythrocyte diaphorase.

METHODS

Preparation of the erythrocyte membranes

The erythrocyte membranes were prepared according to Marchesi et al. (13). Washed human erythrocytes were hemolysed by 30 vol of 5 mM Tris-HCl, pH 7.5, containing 1 mM EDTA, and the 22,000-*g* pellet was extensively washed in the same medium to obtain a completely white membrane preparation. For the preparation of inside-in resealed ghosts, we used the procedure of Steck and Kant (14) in which hemolysis is initiated by mixing 1 vol of washed erythrocytes with 40 vol of 5 mM sodium phosphate buffer, pH 8, containing 1 mM $MgSO_4$.

Extraction

With detergent. The ghosts were suspended in a 10 mM Tris-HCl buffer, pH 7.4, containing 2% Triton X-100. After 30 min of incubation at 4°C, the suspension was frozen and thawed three times and centrifuged for 10 min at 105,000 *g*, at 4°C, in a Beckman Airfuge (Beckman Instruments Inc., Fullerton, Calif.) centrifuge to spin down small volumes (170 μl). The supernatant was assayed for enzyme activity.

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TABLE I
Effect of Triton X-100 on Erythrocyte Methemoglobin Reductase

	Without Triton X-100	With Triton X-100
Crude unspun hemolysate, *nmol/min/mg Hb*	2.5±0.5	4.8±0.6
Washed erythrocyte membranes, *nmol/min/mg protein*	3.0±0.5	78±10

In both cases the enzyme was assayed according to Hegesh et al. (15). The detergent-treated hemolysate and the detergent-treated membranes were incubated 30 min at 4°C with 2% Triton X-100 (final concentration) before the assay.

With cathepsin D. A 1:1 suspension of membranes in 0.1 M Tris-maleate, pH 5.6, was incubated for 2 h at 37°C with cathepsin D (5 μg/10 mg membrane protein). The preparation was then centrifuged at 105,000 *g* as described above. The supernatant was assayed for enzyme activity.

Preparation of membrane-free hemolysate

Washed erythrocytes were hemolysed with 4 vol of bidistilled water and centrifuged at 105,000 *g* for 10 min at 4°C in the Beckman Airfuge centrifuge. The supernatant contained the soluble enzyme.

Preparation of semipurified soluble cytochrome b_5 reductase from human erythrocytes

The membrane-free hemolysate was diluted with 2 vol of a 5 mM potassium phosphate buffer, pH 7.0, and adjusted to the same pH. Hemoglobin was removed from the diluted hemolysate by a DEAE-cellulose 52 batchwise treatment in the same buffer using 2 g of preswollen resin/ml of packed erythrocytes. The resin was extensively washed in a Bucher funnel with the buffer until the effluent was colorless. The enzyme was eluted with 50 mM potassium phosphate buffer, pH 5.8 that contained 0.1 mM EDTA and 0.3 M KCl, and precipitated by $(NH_4)_2SO_4$ added to 60% saturation. The precipitate was separated by centrifugation and dialyzed against a Tris-HCl buffer 10 mM, pH 7.5, overnight at 4°C for enzyme assays. This procedure yielded a hemoglobin-free preparation of the soluble cytochrome b_5 reductase.

Enzyme assays

All assays were carried out at 25°C. The NADH-methemoglobin reductase activity was assayed with ferrocyanide methemoglobin complex as an acceptor, according to Hegesh et al. (15).

The NADH-diaphorase activity was assayed with three different xenobiotic electron acceptors: (*a*) dichlorophenol indophenol (1), (*b*) potassium ferricyanide (16), (*c*) 3-(4,5-dimethyl thiazolyl-2)-2,5 diphenyl tetrazolium bromide (MTT)[1] (17).

Acetylcholinesterase was assayed according to the method of Ellman et al. (18); glyceraldehyde-3-phosphate dehydrogenase was assayed according to Beutler et al. (19).

Proteins were estimated by the method of Lowry et al. When large amount of Triton X-100 produced a precipitate in the final mixture, it was discarded by 5-min centrifugation at 10,000 rpm.

Electrophoresis

Horizontal starch gel electrophoresis was performed as described by Kaplan and Beutler (20) using a Tris-EDTA borate buffer, pH 8.6. Polyacrylamide gel isoelectrofocusing was carried out in a pH 6–8 linear gradient (Ampholines; LKB Instruments, Inc., Orsay, France) according to the method of Drysdale et al. (21). In all the electrophoretic studies specific staining for NADH-diaphorase activity was performed with a mixture containing 1.2 mM NADH, 0.06 mM dichlorophenol indophenol, and 1.2 mM MTT in a 0.25 M Tris-HCl buffer (pH 8.4) (20).

Immunological studies

Inactivation by antiserum. The different preparations were incubated with a chicken antiserum prepared against human erythrocyte-soluble cytochrome b_5 reductase (8) in the presence of 5% (vol/vol) of polyethylene glycol. Increasing amounts of antiserum were added to constant amounts (expressed as units of methemoglobin-ferrocyanide reducing activity) of enzyme. After incubation for 1 h at 4°C followed by centrifugation (20 min at 22,000 *g*) the residual activity was measured in the supernatant.

Double immunodiffusion. Double immunodiffusion was performed according to Ouchterlony (22). Antigens and antiserum were incubated for 2–3 d and then the plate was extensively washed with isotonic saline solution for 2 d to remove the excess enzyme that had not reacted with the antiserum. The precipitation lines were specifically visualized using the NADH-diaphorase staining method (20).

Chemicals

NADH, 2,6-dichlorophenol indophenol, MTT, Triton X-100, potassium ferricyanide, cathepsin D, D-L glyceraldehyde-3-phosphate, adenosine 5-triphosphate, 5,5 dithiobis-(2 nitrobenzoic acid), acetyl thiocholine iodide were from Sigma Chemical Co., St. Louis, Mo. DEAE-cellulose 52 was from Whatman Inc., Clifton, N. J.

RESULTS

Erythrocyte membrane methemoglobin reductase activity. Treating a noncentrifuged 1:4 hemolysate by 2% Triton X-100 (final concentration) doubles the activity of the enzyme assayed by the method of Hegesh (15) (Table I).

The detergent had no effect on the soluble enzyme present in a membrane-free hemolysate (105,000 *g* supernatant). Therefore, the presence of the enzyme as a component of the erythrocyte membrane was investigated. The washed membranes were found to contain a methemoglobin reductase-like activity that was increased 15-fold by detergent treatment (Table I). In contrast, extensive washing of the membranes by

[1] *Abbreviation used in this paper:* MTT, 3-(4,5-dimethyl-thiazolyl-2)-2,5 diphenyl tetrazolium bromide.

1 M NaCl did not result in the appearance of this enzyme activity in the 105,000 *g* supernatant.

Accessibility of various electron acceptors. The accessibility of each electron acceptor was determined by measuring the enzyme activity with each of them in the absence and presence of detergent. As shown in Table II, the membranes exhibit little accessibility to the ferrocyanide methemoglobin complex (64,000 mol wt) used in the Hegesh assay (15). In contrast, with smaller acceptors, such as ferricyanide, dichlorophenol indophenol, or MTT the accessibility was dramatically increased.

Membrane solubilization by Triton X-100 and detergent/protein ratio. The membranes were suspended in 1 vol of 10 mM Tris-HCl buffer, pH 7.4, and the protein concentration adjusted to 4 mg/ml. Triton X-100 was added to obtain a final concentration varying between 0.05 and 10% (wt/vol). Each preparation was centrifuged for 10 min at 105,000 *g* at 4°C. The supernatant was assumed to contain the solubilized fraction of the membrane. The precipitate contains the insoluble part of the enzyme. The solubilization is dependent on the Triton X-100 concentration and maximal solubilization is reached at 2% Triton (wt/vol). At this concentration 75% of the enzyme activity is recovered in the supernatant, 25% remaining in the precipitate.

Configuration of the erythrocyte membrane preparations. To check the configuration of the membranes prepared by Marchesi's method we have assayed two enzyme markers, one of the inner face of the membrane (glyceraldehyde-3-phosphate-dehydrogenase) and the second of the external face of the membrane (acetylcholinesterase). The membranes that we used were unsealed because both enzymes were found to be equally accessible (94% accessibility). The percentage of accessibility was derived from the following ratio: activity minus detergent vs. activity plus detergent, times 100.

TABLE II

Accessibility to Various Electron Acceptors of the Erythrocyte Membrane Methemoglobin Reductase

Acceptor	Without Triton X-100	With Triton X-100	Accessibility
	nmol/min/mg protein		%
Ferrocyanide methemoglobin complex (mol wt = 64,000)	6.37	82	7.8
Dichlorophenol indophenol (mol wt = 290)	37.4	66	57
MTT (mol wt = 414)	37.5	55.5	68
Ferricyanide (mol wt = 329)	0.409	0.405	100

All enzyme activities determinations were carried out at 25°C. The incubations in the presence of detergent were performed at +4°C with 2% Triton X-100. The percent accessibility is determined by the without Triton X-100/with Triton X-100 ratio × 100.

TABLE III

Solubilization by Cathepsin D of the Erythrocyte Membrane Methemoglobin Reductase

	Methemoglobin reductase activity		
	Pellet + Triton	105,000 *g* supernatant − Triton	Percent solubilized
	nmol/min/mg protein		
Unsealed ghosts − cathepsin D	57	0	0
Unsealed ghosts + cathepsin D	21.3	12.3	36.6
Resealed ghosts (inside-in) + cathepsin D	34	0	0

Ghosts prepared as described in text were incubated for 2 h at 37°C with 0.05% cathepsin D in 0.1 M, Tris-maleate, pH 5.6. Assays were performed on 105,000 *g* supernatants and on pellets. The extent of solubilization of the enzyme is expressed as the percentage of enzyme activity in the supernatant relative to the total values recovered in the supernatant and the pellet.

Solubilization of the membrane-bound cytochrome b_5 reductase by cathepsin D. The ghosts were incubated 2 h at 37°C with cathepsin D at a concentration of 0.05%. After incubation the preparation was centrifuged at 105,000 *g* 10 min. The supernatant contained 36% of the total enzyme activity (Table III). Upon isoelectric electrofocusing in a pH 6–8 gradient the cathepsin D solubilized enzyme migrated as a single band with NADH-diaphorase activity (Fig. 1). The isoelectric point (pI) was 6.5. With the soluble NADH-diaphorase, present in a membrane-free (105,000 *g* supernatant) hemolysate, an isoelectric pH of 6.7 was found (Fig. 1).

It was not possible to solubilize by cathepsin D the membrane-bound enzyme of sealed (inside-in) ghosts (Table III). This experiment shows that the erythrocyte membrane methemoglobin reductase is bound to the inner face of the membrane.

Immunological comparison with soluble erythrocyte cytochrome b_5 reductase. Double immunodiffusion showed that the detergent-treated erythrocyte membrane enzyme and the soluble erythrocyte enzyme react similarly toward an antiserum directed against the soluble erythrocyte cytochrome b_5 reductase. The electrophoretic behavior of the membrane-bound enzyme in starch gel followed by staining for NADH-diaphorase activity was also investigated. As seen on Fig. 2 the membrane enzyme migrates more slowly than the soluble enzyme. However it is completely

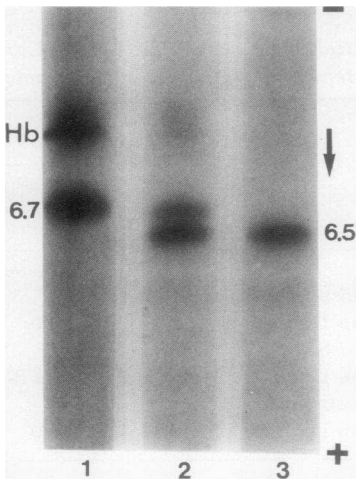

FIGURE 1 Isoelectric focusing in a 6–8 pH gradient (21) followed by specific staining for NADH-diaphorase (20). 1, Membrane free hemolysate; 2, Membrane free hemolysate plus 105,000 *g* supernatant of cathepsin D-treated erythrocyte membrane; 3, 105,000 *g* supernatant of cathepsin D-treated erythrocyte membrane.

abolished by pretreatment with the antiserum directed against the soluble cytochrome b_5 reductase. Under these conditions the soluble enzyme is also completely inhibited (not shown) (8).

Inactivation test by increasing amounts of antiserum was performed upon (*a*) the semipurified soluble cytochrome b_5 reductase in the absence and presence of cathepsin D, (*b*) the detergent-treated membrane enzyme, and (*c*) the cathepsin D-treated membrane enzyme. The inactivation curves obtained were similar in the four cases (Fig. 3).

Status of membrane cytochrome b_5 reductase from patients with recessive congenital methemoglobinemia. We investigated six patients with recessive congenital methemoglobinemia (Table IV). Three patients were without mental retardation (type I disease) and three patients were severely mentally retarded (type II disease) (23, 24 and unpublished cases). In both types the soluble enzyme had been found to be defective (Table IV). Now we have found that the membrane-bound enzyme is also defective in all the patients, but to a different extent. In two of the three patients with the type I disease, the membrane-bound enzyme is not completely defective (Table IV).

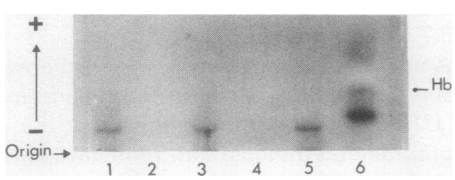

FIGURE 2 Starch gel electrophoresis of detergent-treated membrane erythrocyte enzyme and membrane-free hemolysate. The electrophoresis was performed in 12% starch gel in Tris-EDTA borate, pH 8.6. After migration for 18 h at +4°C, 200 volts the enzyme was stained as NADH-diaphorase (20). Before electrophoresis the membrane enzyme preparations were incubated with identical amounts of saline, normal chicken serum or chicken antiserum directed against human erythrocyte methemoglobin reductase. 1, detergent-treated membranes + NaCl 0.15 M; 2 and 4, detergent-treated membranes plus antiserum; 3, detergent-treated membranes plus normal serum; 5, detergent-treated membranes; 6, membrane-free hemolysate.

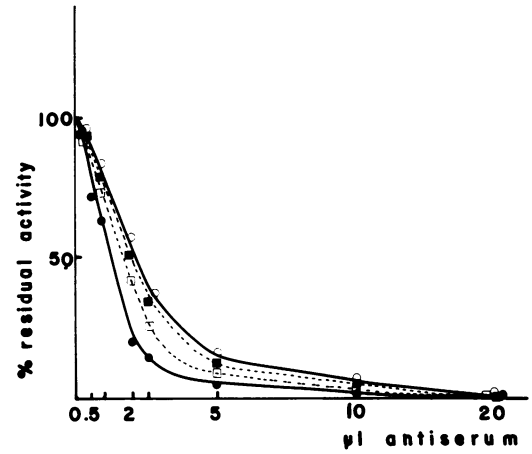

FIGURE 3 Inactivation of human soluble erythrocyte methemoglobin reductase and erythrocyte membrane enzyme by chicken antiserum directed against human soluble erythrocyte methemoglobin reductase. The incubation mixture (60 µl contained 10 µl of enzyme preparation, 10 µl of different dilutions of chicken antiserum, 6 µl of polyethylene glycol (5% final) and 34 µl of saline. Base line enzyme activities were made equal before incubation of antiserum. After incubation for 1 h at 4°C and centrifugation for 30 min at 22,000 *g*, the supernatant was assayed for residual methemoglobin reductase activity according to the method of Hegesh et al. (15). The results are expressed as percentage of the activity measured in control experiments in which antiserum was replaced by normal chicken serum. (□) Semipurified soluble enzyme without cathepsin D; (■) semipurified soluble enzyme plus cathepsin D, 0.05%; (○) detergent (2% Triton X-100) solubilized membrane enzyme; (●) cathepsin D solubilized membrane enzyme.

DISCUSSION

It has been established that the erythrocyte NADH-methemoglobin reductase is a soluble form of cytochrome b_5 reductase (5–8). In the present study we show that the addition of a detergent to the total unspun hemolysate doubles the methemoglobin reductase activity. Inasmuch as the detergent has no activating effect on a membrane-free hemolysate, this phenomenon strongly suggests that the enzyme could be an intrinsic component of the human erythrocyte membrane, as previously proposed by Goto-Tamura et al.

TABLE IV
Membrane-bound and Soluble Methemoglobin Reductase from Patients with Recessive Congenital Methemoglobinemia

Case	Membrane enzyme activity*	Soluble enzyme activity
	nmol/mg protein	*nmol/mg hemoglobin*
Type I disease (without mental retardation)		
CHIR	15	0.3
PIER	0	0.1
BOUR	10	0.5
Type II disease (with mental retardation)		
NASS	0	0
BOU	0.5	0.1
LLE	0	0
Controls	75±10	2.7±0.5

The enzyme activity was assayed according to Hegesh et al. (15).
* Measured in the presence of 2% Triton X-100.

for rabbit erythrocyte ghosts (6). Indeed, thoroughly washed human erythrocyte membranes contain a methemoglobin reductase-like activity that is unmasked by detergent treatment, but cannot be released by high ionic strength treatment. Like the microsomal cytochrome b_5 reductase, which is released as a soluble entity by lysosomal cathepsin (25–27), the erythrocyte membrane-bound enzyme is solubilized by cathepsin D treatment. This treatment is fully effective if ghosts are unsealed, whereas it is ineffective if they are resealed "inside-in." This indicates that the enzyme is actually bound to the inner face of the membrane. In the absence of detergent, the unsealed ghosts exhibit almost no activity on the ferrocyanide-methemoglobin complex. In contrast, with a small substrate such as potassium ferricyanide they exhibit maximal activity even in the absence of detergent. Similarly other small xenobiotic acceptors, such as dichlorophenol indophenol and MTT behave in the same way, whereas their activity was only submaximal in the absence of detergent (57 and 67%, respectively of the maximal activity obtained with detergent). This suggests that the membrane-bound enzyme is more accessible to small substrates than to the much larger molecule of methemoglobin.

The presence of such an enzyme on the inner face of the erythrocyte membrane raises several questions: (*a*) Is it a membrane-bound cytochrome b_5 reductase? (*b*) If so, is it related to the soluble erythrocyte cytochrome b_5 reductase? (*c*) Is it also implicated in the mechanism of methemoglobin reduction? It is possible to give positive answers to questions (*a*) and (*b*). The proteolytic release of the erythrocyte membrane bound enzyme by cathepsin D is reminiscent of similar results obtained with liver microsomes (15, 26). It has been established that the microsomal-bound enzyme is an amphiphilic protein consisting in a hydrophilic moiety carrying the active site and a hydrophobic domain at the COOH-terminal end by which the protein is anchored to the endoplasmic reticulum (28–30). Treatment by cathepsin D causes the cleavage of the molecule at the junction between the hydrophilic and hydrophobic domains and releases an active soluble enzyme (29–32). In the case of the erythrocyte membrane-bound enzyme the site of cleavage by cathepsin D must be different from that of the putative endogenous protease, because of the observed differences in pI between the cathepsin D solubilized enzyme and the spontaneously soluble enzyme (Fig. 1).

Immunological studies confirm the relationship between the erythrocyte membrane-bound enzyme and the soluble cytochrome b_5 reductase. Double immunodiffusion electrophoresis and inactivation curve, after exposure to an antiserum directed against the soluble erythrocyte cytochrome b_5 reductase, show an identical behavior. Because the soluble enzyme has been previously demonstrated to be immunologically similar to the microsomal cytochrome b_5 reductase in human (8) and animals (6, 7), it can be deduced that the enzyme that is strongly bound to the erythrocyte membrane is also a cytochrome b_5 reductase. The finding that in six subjects with congenital recessive methemoglobinemia (type I and type II) the deficiency of erythrocyte-soluble cytochrome b_5 reductase is invariably associated with a deficiency of the erythrocyte membrane enzyme provides an ultimate proof of their common genetic origin.

The identity between the erythrocyte membrane cytochrome b_5 reductase assayed according to Hegesh et al. (15) and the NADH-diaphorase stained by 2,6 dichlorophenol indophenol after electrophoresis (20) is demonstrated by the strict parallelism of the results obtained with the two methods. Conversely the identity between the erythrocyte membrane cytochrome b_5 reductase and the NADH erythrocyte membrane oxidoreductase described by Zamudio (16, 33) is still not demonstrated but is very likely. An atebrin-sensitive NADH-oxidoreductase has been recently described in mouse liver plasma membranes (34). This enzyme should be different from the cytochrome b_5 reductase that is bound to the endoplasmic reticulum and to the erythrocyte membrane since the latter is not affected by atebrin (data not shown).

Regarding the possible role of the membrane enzyme in methemoglobin reduction (question c), we cannot answer yet. It is noteworthy that cytochrome b_5 has already been found as a component of the erythrocyte membrane (35). To what extent the other classical functions of the cytochrome b_5-mediated

electron transport system are operating in the erythrocyte membrane is also still unexplored. Concerning methemoglobin reduction, it should be noted that we have found that, in spite of the absence of detectable soluble methemoglobin-reductase in chicken erythrocytes, the intact cells can promote methemoglobin reduction after exposure to nitrite (unpublished data). Board et al. (36) have found that in nucleated erythrocytes from birds and reptiles the enzyme is only membrane-bound. It is therefore possible that the membrane-bound cytochrome b_5 reductase of the human anucleated erythrocytes does play a role in the reduction of methemoglobin.

Finally, whatever its metabolic role, the membrane-bound erythrocyte cytochrome b_5 reductase seems to represent the primary gene product of the DIA_1 locus (3, 4). This is suggested by its deficiency in recessive congenital methemoglobinemia, which indicates that the erythrocyte membrane cytochrome b_5 reductase is under the same genetic control as the soluble erythrocyte enzyme. Their respective distribution, about half and half, is probably a unique situation proper to the mature circulating erythrocyte. The membrane-bound enzyme could serve as a precursor of the soluble form that would be released by partial proteolysis of the former. Preliminary results, obtained by sucrose gradient centrifugation and high speed gel filtration showed us that the soluble enzyme and the cathepsin D solubilized membrane enzyme display an identical molecular weight lower than that of the membrane bound detergent treated enzyme (manuscript in preparation). A similar model has been suggested by Hultquist (37) for the production of the soluble cytochrome b_5 from its reticulum-bound precursor during the erythroid maturation. Concerning the reductase it would be important to identify the proteolytic enzyme involved in its maturation, and to determine at which stage the post-translational processing of the membrane-bound enzyme occurs. These problems are under current investigation.

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