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A HIGH MOLECULAR WEIGHT FORM OF NADH–CYTOCHROME b_5 REDUCTASE FROM OX LIVER MICROSOMES

E. PANFILI, G. L. SOTTOCASA AND B. DE BERNARD
Istituto di Chimica Biologica, Università degli Studi di Trieste, Trieste (Italy)
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SUMMARY

We report here the solubilisation and purification of NADH–cytochrome b_5 reductase as a high molecular weight species of the order of 400000. As in the procedure reported by Ito and Sato for the isolation of polymer cytochrome b_5, the solubilisation of the enzyme has been performed by means of Triton X-100 and sodium deoxycholate. The purification was then performed through $(NH_4)_2SO_4$ precipitation between 25 and 45 % saturation and gel filtration on Sephadex G-100 and G-50. The last traces of hemoproteins were easily removed by ion exchange chromatography on DEAE-Sephadex A-50. Removal of the phospholipids and detergents is achieved by precipitation with 90 % cold acetone. The enzyme is finally purified by gel filtration on Sephadex G-200. The procedure results in a 208-fold purified enzyme with a 19 % yield. Disc electrophoresis on 3 % polyacrylamide gel of the preparation reveals the presence of a single component which stains positively with periodic acid-Schiff reagent and gives a metachromatic response to toluidine blue. These findings indicate that the enzyme contains carbohydrates. The band does not show positive reaction with dyes specific for lipids. Only upon treatment with urea or sodium dodecyl sulphate is the enzyme split into three subunits.

INTRODUCTION

Properties of microsomal NADH–cytochrome b_5 reductase had been known for a long time as a result of the work of STRITTMATTER'S[1–4] group. The enzyme has been reported to display a molecular weight of the order of 40000. Other preparations of this reductase with similar properties have been recently reported by TAKESUE AND OMURA[5] and by ST. LUIS *et al.*[6]. All these preparations were obtained by enzymatic digestion of microsomes either with *naja-naja* venom or lysosomal enzymes. ST. LOUIS *et al.*[6] concluded that the solubilizing agent in lysosomes is an acid proteinase. In order to avoid proteo- or lipolytic digestions of the membranes, we have attempted to isolate the enzyme by means of detergents. Such an approach had been already used succesfully by ITO AND SATO[7] for the purification of a polymeric form of microsomal cytochrome b_5.

This paper describes in detail the procedure leading to a 208-fold purification

Abbreviation: DCIP, 2,6-dichlorophenolindophenol.

of the enzyme, essentially homogeneous electrophoretically, whose molecular weight is of the order of 400000.

Short reports of this investigation have been already presented[8-10].

METHODS

Enzyme assay

NADH–cytochrome b_5 reductase activity was determined spectrophotometrically essentially according to STRITTMATTER[2], following the cytochrome *c* reduction at 550 nm (Optica CF4 recording spectrophotometer), in the presence of catalytic amount of cytochrome b_5.

NADH–ferricyanide reductase activity was followed spectrophotometrically at 410 nm according to SOTTOCASA *et al.*[11].

NADH–2,6-dichlorophenolindophenol (DCIP) reductase activity was followed spectrophotometrically at 605 nm according to SAVAGE[12].

The millimolar extinction coefficients are respectively 19.1 for cytochrome *c*[13], 1.0 for ferricyanide[14], and 21.0 for DCIP[13].

Cytochrome b_5 used as electron acceptor was prepared in our laboratory by tryptic digestion of beef liver microsomes according to POLTORATSKJ-BOIS AND CHAIX[15]. The hemoprotein has a molecular weight of 13000[16].

Polyacrylamide gel electrophoresis

Electrophoretic behaviour of the purified enzyme has been tested according to the technique of DAVIS[17]. Prerunning of the gels with Tris–HCl–TEMED buffer (pH 8.9) was performed for 2 h at 5 mA per tube to get rid of $(NH_4)_2S_2O_8$ (ref. 18). Lyophilized NADH–cytochrome b_5 reductase samples were equilibrated with the spacer gel buffer by Sephadex G-25 gel filtration column (5 mm × 70 mm), pre-equilibrated with the same buffer. The sample containing 200 μg protein and 40 % sucrose was loaded onto the spacer gel. The electrophoresis was then run at 5 mA in a refrigerator at pH 8.3 as suggested by DAVIS[17]. After 1 h gels were removed and stained for proteins with Amido black. The enzyme activity was detected on parallel gels or on the same gel prior to staining as follows: gels were soaked in 0.25 M phosphate buffer (pH 7.4) containing 0.1 % DCIP and 3 mM NADH. The gels stain blue except in the region where the dye is reduced by the enzyme. Staining for glycoprotein components has been carried out by soaking the gels in 0.1 % toluidine blue in 0.1 M acetate buffer (pH 4.5). After 8–10 h the excess of the dye was removed by immersion in the same buffer till glycoprotein appeared as a pink-violet band, contrasting the virtually uncolored background. The glycoprotein was also stained after periodic acid oxidation with Schiff reagent according to EVANS[19]. Coomassie brilliant blue was used to detect protein when the electrophoresis was carried out in 0.1 % sodium dodecyl sulphate, according to WEBER AND OSBORNE[20]. Lipids were stained by Sudan III in 70 % ethanol saturated solution[21]. Densitometric traces of the gels were obtained by using white light of an Eppendorff spectrophotometer, equipped with a home-made automatic scanning device.

Determination of the molecular weight

The molecular weight of purified NADH–cytochrome b_5 reductase was calculated using both the gel filtration[22] and electrophoretic gel permeation. A Sephadex G-200

column (2.9 cm × 46 cm) equilibrated in 50 mM Tris–HCl buffer (pH 7.5) containing 0.1 M KCl was used. Electrophoretic runs were performed in 3.1 and 7 % polyacrylamide gels (6 mm × 75 mm) at 4 mA per gel at pH 8.3. The standard proteins were: cytochrome *c* from horse heart (Sigma Chem. Co., Type III) molecular weight 12384, purified by electrophoresis in our laboratory; chymotrypsinogen (Boehringer), molecular weight 25000; bovine serum albumine (Boehringer), molecular weight 67000; alcohol dehydrogenase from equine liver (Sigma Chem. Co.) molecular weight 84000; beef liver catalase (Sigma Chem. Co.), molecular weight 230000; yeast hexokinase (Sigma Chem. Co., Type III), molecular weight 100000, and aldolase (Boehringer), molecular weight 147000.

Protein determination

Protein was determined either according to GORNALL *et al.*[23], or, in the case of very diluted protein solutions, according to WARBURG AND CHRISTIAN[24], using the empirical equation of LAYNE[25].

Preparation of microsomes

Beef liver from the slaughterhouse was homogenized at 25 % in 0.25 M sucrose containing 0.1 mM EDTA (pH 7.4) using a commercial blendor for 1 min. Microsomes were obtained from a post-mitochondrial supernatant (8000 × *g* for 20 min, rotor 872, International refrigerated centrifuge, Model B-20) by $(NH_4)_2SO_4$ precipitation at 40 %, according to STRITTMATTER[3].

RESULTS

All the steps indicated in this chapter were carried out at 1–5°, unless otherwise stated.

Step 1. Solubilization of NADH–cytochrome b_5 reductase

Dialyzed microsomes in 50 mM Tris–HCl buffer (pH 7.4) were added with Triton X-100 and sodium deoxycholate to a final concentration of 1 % of each detergent and of about 25 mg protein per ml. After standing for 30 min under stirring, solid $(NH_4)_2SO_4$ was added to 25 % saturation. The precipitate was discarded and a fraction was collected by centrifugation after addition $(NH_4)_2SO_4$ up to 45 % of the saturation value. Upon centrifugation in a No. 870 rotor on the International refrigerated centrifuge at 15000 × *g* for 20 min, the fraction appeared as a red floating pellicle. The bulk of the reductase activity was concentrated in this fraction, which collects also the bulk of polymer cytochrome b_5[7].

Step 2. Gel-filtration chromatography on Sephadex G-100 and G-50

The fraction containing the enzyme was first dialyzed overnight against a solution containing 20 mM Tris–HCl buffer (pH 7.4), 50 mM KCl, 1 mM EDTA and 0.4 % sodium deoxycholate. The dialyzed material was subjected to high speed centrifugation (78000 × *g* for 30 min) using a No. 40 Spinco rotor. The clear supernatant was loaded on a Sephadex G-100 column (8 cm × 32 cm) pre-equilibrated with the dialysis solution. The elution of the column was carried out with the same buffer, and its pattern is illustrated in Fig. 1. It may be seen that the enzyme is eluted

as a rather broad band partially overlapped by a large protein peak, but completely resolved from a delayed protein component, devoid of enzyme activity. The latter peak may be shown to contain the polymeric cytochrome b_5 described by ITO AND SATO[7], which may be obtained as a by-product of this preparation. The enzyme containing fractions, pooled, were subjected to gel filtration on a Sephadex G-50 column (5 cm × 125 cm), pre-equilibrated with a solution containing 20 mM Tris–HCl buffer (pH 7.4), 2 mM EDTA and 1 % Triton X-100. The purpose of this chromatography is to replace sodium deoxycholate, which is retained by the gel, with Triton X-100. All the proteins are eluted from the column as a single peak with the void volume.

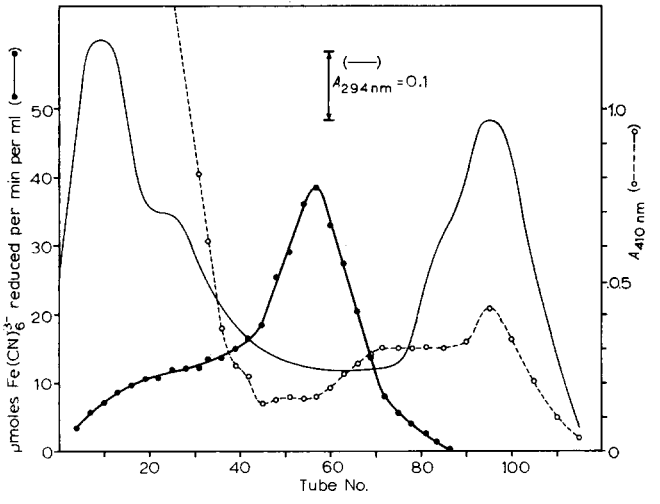

Fig. 1. Elution pattern from Sephadex G-100 column (8 cm × 32 cm) of the 25–45% precipitate with $(NH_4)_2SO_4$. Experimental conditions: the chromatography was carried out with 20 mM Tris–HCl buffer (pH 7.4), containing 50 mM KCl, 1 mM EDTA and 0.4% sodium deoxycholate. Fractions 39–69 were pooled and further processed. ———, protein absorbance; ○---○, hemoprotein absorbance; ●—●, enzymic activity.

Step 3. Ion-exchange chromatography

The enzyme-containing fractions obtained by the previous chromatography were combined and subjected to a DEAE Sephadex A-50 column (4.5 cm × 15 cm) pre-equilibrated with a solution containing 20 mM Tris–HCl (pH 7.4), 2 mM EDTA and 0.5 % Triton X-100. Under these conditions NADH–cytochrome b_5 reductase is firmly bound to the ion exchanger and so does cytochrome b_5. A number of other proteins including hemoglobin are not retained by the resin, and are therefore washed out by the buffer. Washing of the column is performed until the effluent becomes colorless. The enzyme is then displaced by using an exponential KCl gradient from 0 to 0.1 M. NADH–cytochrome b_5 reductase is eluted as a single peak. Even at the highest KCl concentration (0.1 M) cytochrome b_5 is still retained on the ion exchanger. This allows the removal of the last traces of cytochrome b_5 from the enzyme preparation. Fig. 2 shows the elution pattern from DEAE-Sephadex A-50 by the KCl gradient. The enzyme is eluted as a single peak. The optical density increase at 410 nm accompanying the enzyme may be attributed to end-absorption of the flavin moiety of the enzyme. The enzyme containing fractions were combined and dialyzed overnight against 20 mM Tris–HCl buffer (pH 8.5) to remove KCl.

Step 4. Aqueous acetone treatment

It was found that a high protein concentration was required in order to obtain a good precipitation by acetone. In some experiments, therefore, the dialysate was concentrated by ultrafiltration on a Diaflo UM-2 membrane. To 1 vol. of the enzyme solution 9 vol. cold acetone at $-10°$ were slowly added. As soon as a faint flocculation appeared, the mixture was spun down at 8000 $\times$ *g* for 20 min in No. 872 rotor of the International refrigerated centrifuge. The yellowish precipitate was suspended in a very small volume of 100 mM Tris–HCl buffer (pH 7.8) containing 100 mM KCl,

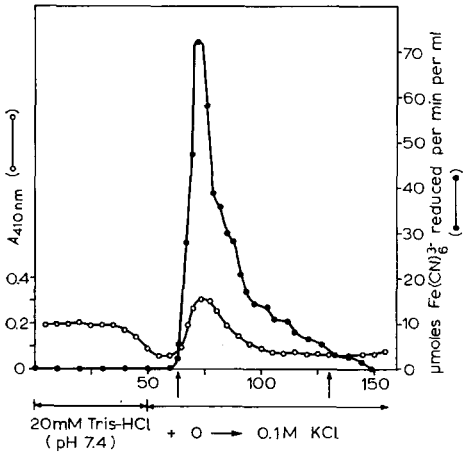

Fig. 2. Elution pattern from DEAE-Sephadex A-50 (4.5 cm $\times$ 15 cm). Experimental conditions. The eluate from Sephadex G-50 was loaded on the column and washed with 20 mM Tris–HCl buffer (pH 7.4) containing 2 mM EDTA and 0.5% Triton X-100. Elution was performed with an exponential KCl gradient from 0 to 0.1 M in the same buffer. Fractions 63–130 were collected. ○—○, hemoprotein absorbance; ●—●, enzymic activity.

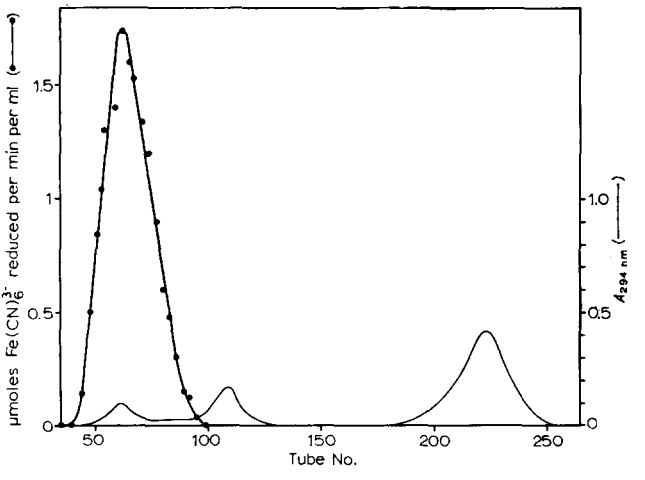

Fig. 3. Elution pattern from Sephadex G-200 column (7 cm $\times$ 53 cm) of aqueous acetone precipitate. Experimental conditions: the elution was performed with 100 mM Tris–HCl buffer (pH 7.8) containing 100 mM KCl, 1 mM EDTA, 1 mM Mg^{2+} and 0.1 mM 2-mercaptoethanol. Fractions 41–90 were pooled and lyophilized. ———, protein absorbance; ●—●, enzymic activity.

1 mM Mg^{2+}, 1 mM EDTA, 0.1 mM 2-mercaptoethanol and centrifuged in No. 40 Spinco rotor at 78000 × *g* for 15 min. The precipitate was discarded, and the clear yellow supernatant was subjected to the last chromatographic purification, as described in the next step.

Step 5. gel-filtration chromatography on Sephadex G-200

A Sephadex G-200 column (7 cm × 53 cm) was equilibrated with the buffer mentioned at the Step 4. This buffer system was chosen in order to preserve the –SH groups and Mg^{2+} of the enzyme[2]. The elution profile is presented in Fig. 3, and indicates that the enzyme behaves as a high molecular weight component, resolved from other inactive proteins. The peak with enzymatic activity, collected and lyophilized, represents the purified NADH–cytochrome b_5 reductase preparation.

Table I summarizes the results of a typical purification of the enzyme from ox liver microsomes. The overall purification is 208 times with a yield of 19 %.

TABLE I

PURIFICATION OF NADH–CYTOCHROME b_5 REDUCTASE FROM OX LIVER MICROSOMES

Fraction	*Total protein (mg)*	*Enzyme activity (μmoles cyt. c reduced per min)*		*Yield (%)*	*Purification (-fold)*
		per mg protein	*Total*		
Microsomes	20 000	0.337	6750	—	—
Microsomes + Triton + sodium deoxycholate	20 000	0.340	6800	100	—
25–45% satd. $(NH_4)_2SO_4$	14 500	0.428	6200	91	1.3
Sephadex G-100	2 640	2.120	5600	82	6.3
DEAE-Sephadex A-50	615	6.750	4140	61	20.0
Acetone ppt.	50	54.000	2700	40	160.0
Sephadex G-200	18	70.000	1280	19	208.0

Electrophoretic behaviour of NADH–cytochrome b_5 reductase

Fig. 4 shows the densitometric traces obtained after polyacrylamide gel electrophoresis of the enzyme. Trace a in the figure has been obtained by amido black staining and it may be noticed that only a minor contaminant is present in the gel. Associated with the high peak it was possible to show the enzyme activity by the reduction of DCIP in the presence of NADH (see METHODS). As shown by traces b and c, the enzyme is associated with material which stains metachromatic with toluidine blue and periodic acid Schiff reagent. These findings suggest that the enzyme contains carbohydrates. On the contrary, no positive response has been obtained with Sudan III, indicating the absence of appreciable amount of lipids. Disc electrophoresis on polyacrylamide gel has therefore revealed that the enzyme migrates as a single band when the gel pores are maintained at a size compatible with a high molecular weight component. This condition is fullfilled by 3 % acrylamide gels. If the gel pores are far smaller (*e.g.* 7 % polyacrylamide), under the severe sieving

effect, the enzyme is split into different bands, most of which still displaying reductase activity.

Molecular weight of NADH–cytochrome b_5 reductase

The graph reported in Fig. 5 clearly indicates that the enzyme obtained by this procedure has a very high molecular weight, which may be extrapolated in the vicinity of $4 \cdot 10^5$. This result is confirmed by electrophoretic gel permeation data as reported in Fig. 6.

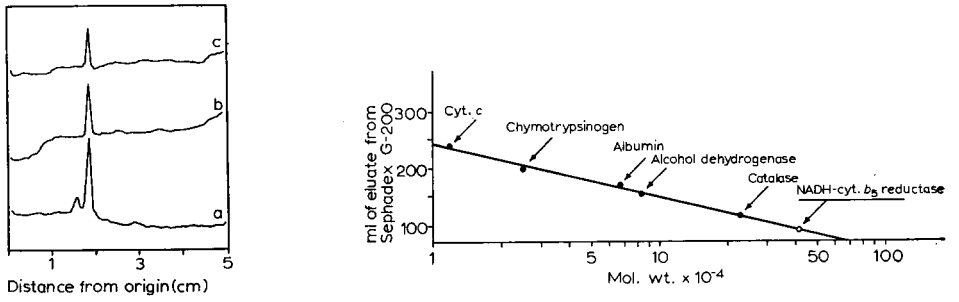

Fig. 4. Electrophoretic pattern of purified NADH–cytochrome b_5 reductase. Experimental conditions: see METHODS. Trace a, Amido black staining; Trace b, periodic acid–Schiff reagent treatment; Trace c, toluidine blue staining.

Fig. 5. Molecular weight of NADH–cytochrome b_5 reductase. Experimental conditions: see METHODS. Elution was followed at 280 nm for chymotrypsinogen and bovine serum albumin (albumin) and at 413 nm for cytochrome *c*; alcohol dehydrogenase was detected according to MARTIN AND AMES[27]; catalase was tested by polarographic measurement of O_2 in presence of catalytic amount of H_2O_2 and NADH–cytochrome b_5 reductase by ferricyanide reduction[11].

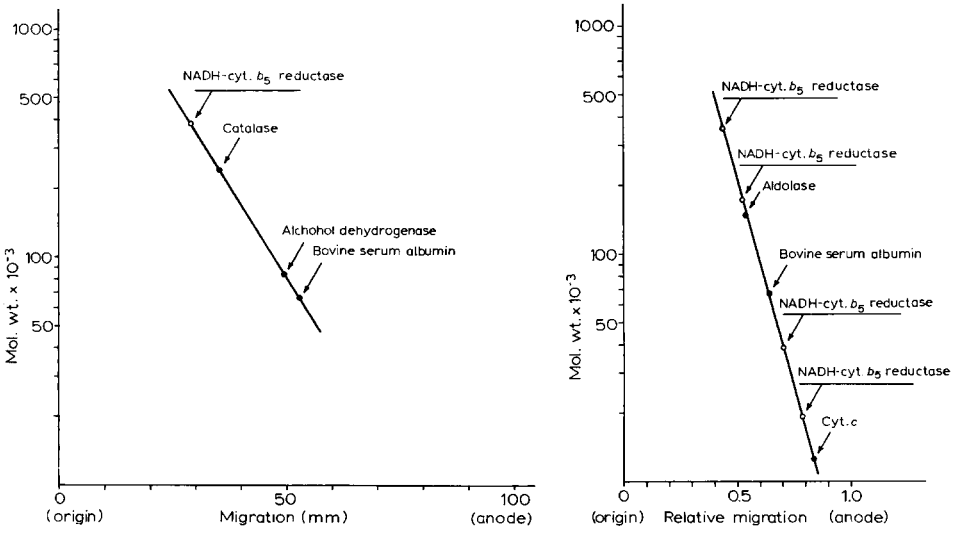

Fig. 6. Molecular weight of NADH–cytochrome b_5 reductase determined by electrophoretic gel permeation. Experimental conditions: see METHODS. Staining was performed with Amido black.

Fig. 7. Dissociation in subunits of NADH–cytochrome b_5 reductase by means of electrophoresis on 7% acrylamide gel in presence of 0.1% sodium dodecyl sulphate. Experimental conditions: see METHODS. Staining was performed with Coomassie blue.

Dissociation in subunits of NADH–cytochrome b_5 reductase

It is expected that if the enzyme is an aggregated form of subunits, dissociative treatment should result in a drastic change of molecular weight of the active species. Splitting of the protein has been obtained by polyacrylamide gel electrophoresis in the presence of 5 M urea. However, better results have been achieved by sodium dodecyl sulphate gel electrophoresis[19]. Results of this experiment are reported in Fig. 7. The smallest subunit has a molecular weight of 19000. The most abundant one gives a value of 39000, which should represent the dimer form of the enzyme. Two other bands are also consistently present, though to a smaller extent, corresponding to molecular weights of 170000 and 360000, respectively.

DISCUSSION

As far as we know, this is the first report concerning the isolation and the purification of NADH–cytochrome b_5 reductase in a polymeric form. From about 1 kg of ox liver as starting material, 15–20 mg of enzyme protein are obtained, the time required for the total process being about 10 days. Our enzyme preparation displays a molecular weight of 400000, a value surprisingly high as compared to that of approximately 40000–50000 reported for other preparations[2,26] of the same enzyme obtained by digestion. Only upon treatment with sodium dodecyl sulphate is the enzyme activity found to be associated with a subunit having a molecular weight of 39000. It appears then that the solubilization of the enzyme by detergents yields a product which tends to aggregate. This phenomenon may or may not be related to the fact that this protein contains a glucidic moiety, which has not been described in the case of the enzyme preparations hitherto reported[2,26]. It is interesting mentioning in this connection that a cytochrome b_5 with a molecular weight of 120000 has been recently obtained from microsomes by means of detergents[7].

Some kinetic parameters of NADH–cytochrome b_5 reductase appear to be independent of the aggregation state of the protein. Similarly to the enzyme described by STRITTMATTER[2], this preparation, obtained from rat liver microsomes, displays a K_m for monomeric cytochrome b_5 of $2.0 \cdot 10^{-5}$ M, and the v_{max} obtained with ferricyanide and DCIP are some 4 times higher than with cytochrome b_5. The enzyme is competitively inhibited by pyrophosphate, and, as expected, does not react with cytochrome *c*. In contrast with Strittmatter's enzyme, however, if polymer cytochrome b_5 is used as the electron acceptor, a v_{max} virtually identical with that obtained with ferricyanide and DCIP is obtained (unpublished results). The latter phenomenon would suggest that the polymeric form of cytochrome b_5 is a more "physiological" substrate for the polymeric enzyme. Presumably the tendency to aggregate both of the NADH–cytochrome b_5 reductase and of the cytochrome b_5 when prepared with detergents, reflects a property of the native forms. It cannot be excluded however, that the binding sites involved in the polymerization, in the intact membrane are occcupied by other membrane components, so that monomers are present in the endoplasmic reticulum and polymers are obtained after removal of phospholipids and other proteins. The aggregated form should in this case be considered artifactual, but would reflect the integrity of the original catalytic protein.

A more detailed study of the chemical structure of the enzyme and its kinetic parameters is now under way in our laboratory.

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