Biochimica et Biophysica Acta, 524 (1978) 262—276

BBA 68450

INTRAMEMBRANEOUS LOCALIZATION OF RAT LIVER MICROSOMAL HEXOSE-6-PHOSPHATE DEHYDROGENASE AND MEMBRANE PERMEABILITY TO ITS SUBSTRATES

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(Received November 24th, 1977)

Summary

A method for purifying hexose-6-phosphate dehydrogenase (β-D-glucose: $NAD(P)^+$ 1-oxidoreductase, EC 1.1.1.47) from rat liver microsomes is described. The purified enzyme was shown to be homogeneous by sodium dodecyl sulfate (SDS)-polyacrylamide electrophoresis.

It is shown that the enzyme is bound to the inner surface of microsomal membranes, and that glucose 6-phosphate, but not NADP, penetrates almost freely into the membranes at 37°C.

Introduction

The physiological role of hexose-6-phosphate dehydrogenase (β-D-glucose: $NAD(P)^+$ 1-oxidoreductase, EC 1.1.1.47) has not yet been fully explored. It seems to be functioning in vivo as a glucose-6-phosphate dehydrogenase [1] and might possibly be involved in the microsomal electron transport system [2], but further elaboration is still required for elucidating its physiological importance.

We have previously reported that rat liver microsomes isolated in isotonic sucrose exhibited no hexose-6-phosphate dehydrogenase activity, but the enzyme was activated by various membrane perturbants, such as detergents and lipid hydrolases [3]. In the present report, we deal with the reason for this latency of this enzyme.

Materials and Methods

Animals and reagents. Male rats (Wistar, 270—330 g) and an adult rabbit were used. 2-Deoxyglucose 6-phosphate, galactose 6-phosphate, NADPH, cytochrome *c* (Type III), L-α-phosphatidylcholine (Type III-E), β,γ-dipalmitoyl-

L-α-phosphatidylethanolamine, phospholipase A from *Crotalus terrificus terrificus* venom, phospholipase C (Type I), trypsin (Type I), Triton X-100 and phenolphthalein glucuronide (Na^+ salt) were purchased from Sigma; inosine diphosphate from Boehringer; NADP and glucose 6-phosphate from Kyowa Hakko Kogyo Co.; sodium deoxycholate from Difco; CM-Sephadex C-50 and 5′-AMP-Sepharose 4B from Pharmacia Fine Chemicals; Freund's complete adjuvant from Iatron and 8-anilino-naphthalene-1-sulfonic acid (ANS) from Tokyo Kasei. All other reagents were commercial products of the highest grade available.

Preparation of microsomes. Rat livers were homogenized in 9 vols. 0.25 M sucrose and microsomes isolated as described previously [3]. The microsomal pellets were suspended in 0.25 M sucrose (14—17 mg protein/ml) and kept at 0—4°C until use. For the purification of hexose-6-phosphate dehydrogenase, the microsomal pellets were stored at —70°C until use.

Enzyme assays. Reaction mixture for hexose-6-phosphate dehydrogenase contained, in 1.0 or 3.0 ml, Tris · HCl (I = 0.04, pH 7.5)/0.24 mM NADP/1.2 mM glucose 6-phosphate or deoxyglucose 6-phosphate or galactose 6-phosphate and enzyme. Reaction was started by the addition of hexose 6-phosphate and extinction changes at 340 nm were measured at 21°C or 37°C. One unit of activity was defined as the amount of the enzyme that reduced 1 μmol of NADP per min.

Glucose-6-phosphatase (EC 3.1.3.9) activity was assayed by a modification of the method of Nordlie and Arion [4]. The assay mixture contained (0.8 ml): 0.4 ml 0.1 M Tris/maleate buffer (pH 6.5); 0.1 ml 120 mM glucose 6-phosphate or deoxyglucose 6-phosphate or galactose 6-phosphate; water (as required) and enzyme. Under the reaction conditions used, the release of P_i was directly proportional to enzyme concentration and also to incubation time. The reaction was stopped by the addition of 0.2 ml 30% trichloroacetic acid and the supernatant was assayed for P_i by the method of Fiske and SubbaRow [5].

NADPH-cytochrome *c* reductase (EC 1.6.2.3) activity was measured at 21°C using 50 mM Tris · HCl buffer/0.16 mM NADPH/0.05 mM cytochrome *c*/1 mM KCN (pH 7.5) in 1 ml. The reduction of cytochrome *c* was followed at 550 nm. The extinction coefficient of $18.5 \cdot 10^3$ $mol^{-1} \cdot l^{-1} \cdot cm^{-1}$ at 550 nm for reduced minus oxidized cytochrome *c* [6] was used.

Nucleoside diphosphatase (EC 3.6.1.6) activity was assayed by the method of Kuriyama [7], except that the incubation time was increased to 10 min. β-Glucuronidase (EC 3.2.1.31) was assayed by the method of Fishman et al. [8] and was expressed as nmol phenolphthalein released per min per mg protein. The concentration of phenolphthalein was estimated using $\epsilon = 26.6 \cdot 10^3$, pH 10.2, 500 nm [9]. The incubation mixture contained 0.8 ml 0.1 M acetate buffer (pH 4.5), 0.1 ml 10 mM phenolphthalein glucuronide, 30 μl microsomal suspension and 70 μl water.

Effects of Triton X-100 were examined with microsomes suspended in 0.4% Triton X-100/0.25 M sucrose. The effects of KCl and sucrose were examined by incorporating these substances in the buffer at concentrations of 0.154 and 0.25 M, respectively.

Protein was determined according to Lowry et al. [10] with bovine serum albumin as a standard.

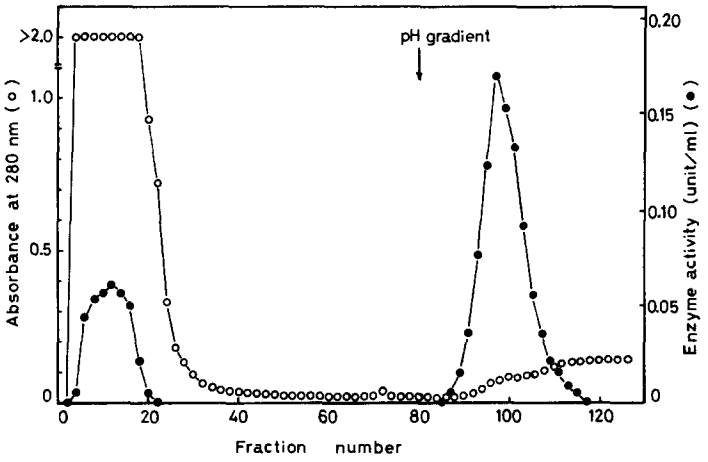

Fig. 1. Chromatography of hexose-6-phosphate dehydrogenase on CM-Sephadex. The dialyzed solution after ammonium sulfate fractionation was placed on a CM-Sephadex column (45 × 270 mm) and the enzyme was eluted by increasing pH. Activity was assayed at pH 7.5 with NADP and deoxyglucose 6-phosphate as substrates. Fractions 91—104 were pooled and concentrated for further purification.

Electrophoresis and molecular weight determinations. Polyacrylamide gel electrophoresis was performed as previously described [3]; SDS electrophoresis was performed according to Weber and Osborn [11]. Standard proteins in the range of 40 000—300 000 daltons were utilized.

Purification of hexose-6-phosphate dehydrogenase. Frozen microsomal pellets were suspended in 6 vols. 20 mM Tris · HCl buffer/1 mM EDTA (pH 7.5) with the aid of a Potter-Elvehjem glass homogenizer, mixed with 0.5% deoxycholate (1/9 vol. 5% (w/v) sodium deoxycholate) and allowed to stand at 2°C for 30 min. The mixture was treated with 1 vol. 3.78 M $(NH_4)_2SO_4$ for 1 h. The precipitate was collected by centrifugation, dissolved in 40 mM phosphate buffer/1 mM EDTA/1 mM iodoacetamide (pH 6.4) and dialyzed overnight against the same buffer. After removal of insoluble materials by centrifugation, the dialysis was completed using an Amicon hollow fiber concentrator (Model CH3, HICX50 cartridge). The dialyzed solution was applied to a CM-Sephadex column (45 × 270 mm) equilibrated in 40 mM phosphate buffer/1 mM EDTA (pH 6.4) and the enzyme eluted with a linear pH gradient (1200 ml) of 40 mM phosphate buffer/1 mM EDTA (pH 6.4—7.3) (flow rate = 120 ml/h; 20-ml fractions; Fig. 1). The pooled fractions of highest activity (pH 6.8) were concentrated (Amicon ultrafiltration cell, XM-50 membrane) and diluted with 1 vol. water. The enzyme was applied to a column of 5′-AMP-Sepharose 4B (22 × 57 mm) equilibrated with 20 mM phosphate buffer/1 mM EDTA (pH 6.8). The column was washed with 20 mM phosphate buffer/1 mM EDTA (pH 6.8) and then with the same buffer containing 0.15 M NaCl (flow rate = 30 ml/h; 4-ml fractions). The enzyme was eluted with the same buffer containing 1 mM NAD (pH 6.8). The peak fractions having the same specific activity were combined (Fig. 2). For immunological studies, the purified enzyme was dialyzed against phosphate-buffered saline and concentrated using a collodion bag (Sartorius Membranfilter Co., Göttingen).

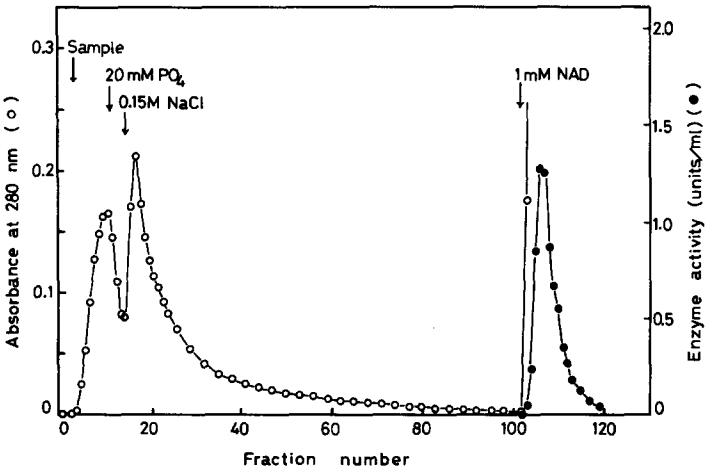

Fig. 2. Chromatography of hexose-6-phosphate dehydrogenase on 5'-AMP-Sepharose 4B. The concentrated solution from CM-Sephadex chromatography was diluted with 1 vol. distilled water, and placed on 5'-AMP-Sepharose 4B column (22 X 57 mm). The enzyme was eluted with 1 mM NAD/20 mM phosphate buffer/0.15 M NaCl/1 mM EDTA, pH 6.8. Activity was assayed as in Fig. 1. Fractions 105—109 were pooled.

Antibody preparation. Antibody to the purified enzyme was raised in an albino rabbit using Freund's complete adjuvant. The serum was diluted with 1 vol. phosphate-buffered saline and fractionated with $(NH_4)_2SO_4$ at 40% saturation. The precipitate was dissolved in distilled water and again fractionated with $(NH_4)_2SO_4$ at 33% saturation. The resulting precipitate was dialyzed against 20 mM phosphate buffer/0.15 M NaCl/1 mM EDTA (pH 6.8). The antibody thus prepared formed a single precipitin line with the purified enzyme upon agar diffusion, but an additional faint line was observed when tested against deoxycholate-solubilized microsomes. Therefore, the antibody was further purified according to the procedure reported by Yunis et al. [12] for the purification of goat anti-phosphorylase antibody. This antibody preparation contained 2.41 mg protein and inhibited 1.58 units of enzyme per mg protein (about 40% recovery). Upon agar diffusion test, the antibody gave a single connecting precipitin line with both pure and crude enzyme samples.

Ouchterlony agar diffusion was performed at room temperature in 1.5% agarose gel/25 mM Tris/maleate buffer/50 mM NaCl/0.5 mM EDTA (pH 7.5).

Fluorescence measurement. Fluorescence was measured at 16—44°C with a Hitachi fluorescence spectrophotometer (204-S) equipped with a 150-W Xenon lamp and a Xenon lamp power supply. To 3 ml 5 mM Tris · HCl buffer/0.1 mM 8-anilino-naphthalene-1-sulfonic acid (ANS) (pH 7.6), with or without 0.25 M sucrose, was added 10—20 μl intact (or phospholipase C-treated or trypsin-treated) microsomes, or 50 μl phospholipid micelles. The mixture was then equilibrated at 16°C for 20 min, and change of fluorescence was recorded by increasing temperature at a rate of about 0.25°C per min. The cells was constantly agitated during assay so as to minimize the distributional difference of temperature within the cell. Micellar dispersions of phospholipids were prepared by sonicating phosphatidylcholine and phosphatidylethanolamine (2 mg/ml) with 5 mM Tris · HCl buffer (pH 7.6).

Results

Stability and molecular weight of hexose-6-phosphate dehydrogenase

Table I summarizes the results of a typical purification experiment. The enzyme thus purified was electrophoretically homogeneous, but was immunologically heterogeneous.

The purified enzyme lost about 25% of its activity during 30 days of storage at 0—4°C, pH 6.8. Such loss of activity could not be prevented by the following substances: glucose 6-phosphate, NAD, NADP and 2-mercaptoethanol (1 mM); phosphatidylcholine (6 μg per μg of pure enzyme); phosphatidylethanolamine (30 μg per μg of pure enzyme); glycerol (50%); deoxycholate (0.05%); and Triton X-100 (0.1%). $(NH_4)_2SO_4$, which has been found to be a potent stabilizer of starfish hexose-6-phosphate dehydrogenase [13], had no effect on rat hexose-6-phosphate dehydrogenase.

The molecular weight of hexose-6-phosphate dehydrogenase was estimated by polyacrylamide gel electrophoresis at 190 000 and 105 000 in the presence of SDS. In agreement with our previous data with crude enzyme samples, this suggests that the enzyme is composed of two identical subunits of molecular weight approx. 100 000.

Hexose-6-phosphate dehydrogenase has been known to degrade upon tryptic digestion into two enzymatically active smaller molecules [14,15]. In fact, the tryptic digestion of purified hexose-6-phosphate dehydrogenase yielded first an active molecule of molecular weight 144 000 and then an active molecule of molecular weight 115 000. These values coincide with the previous values obtained with crude enzyme samples [14].

Effect of antibody on purified hexose-6-phosphate dehydrogenase

Fig. 3 illustrates typical neutralization curves obtained when increasing amounts of antibody were added to a given amount of purified hexose-6-phosphate dehydrogenase. The enzyme activity was assayed at pH 7.5 with either glucose 6-phosphate or deoxyglucose 6-phosphate or galactose 6-phosphate as substrate. As is evident in this figure, the activity on deoxyglucose 6-phosphate and galactose 6-phosphate was inhibited progressively by increasing amounts of antibody, whereas the activity on glucose 6-phosphate was stimulated about 1.4-fold in an antigen excess area. Interesting was the finding that identical neutralization curves were also obtained with the trypsin-digested enzyme having a molecular weight of 115 000. This might not necessarily mean that the

TABLE I

PURIFICATION OF MICROSOMAL HEXOSE-6-PHOSPHATE DEHYDROGENASE

Purification was carried out as described in the text. Activity was assayed at pH 7.5 with NADP and deoxyglucose 6-phosphate as substrates.

	Total protein (mg)	Enzyme activity		
		Total units	Units/mg	Yield
Deoxycholate-treated suspension	6532	57.3	0.009	100
Ammonium sulfate	2142	43.4	0.020	75.7
CM-Sephadex	17.7	30.7	0.17	53.6
5'-AMP-Sepharose 4B	2.1	18.9	9.0	33.0

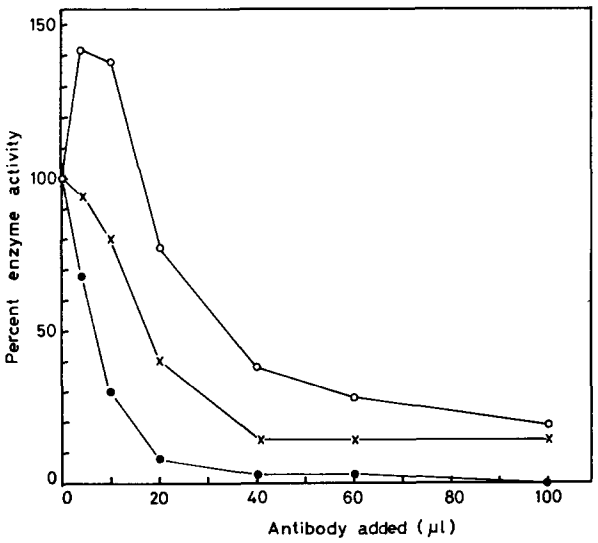

Fig. 3. Effect of antibody on the activity of purified hexose-6-phosphate dehydrogenase. Purified enzyme preparations were incubated with antibody for 20 min at 21°C in 50 mM Tris · HCl, pH 7.5, and then the enzyme activity was assayed at 21°C by the addition of NADP and one of the substrates. The substrates were glucose 6-phosphate (○), deoxyglucose 6-phosphate (●), and galactose 6-phosphate (X). The activities toward glucose 6-phosphate, deoxyglucose 6-phosphate and galactose 6-phosphate in the absence of antibody were 2.1, 9.2 and 2.8 munits, respectively. These activities were not affected by normal γ-globulin.

enzymatically inactive fragments formed upon tryptic digestion of hexose-6-phosphate dehydrogenase would not have any antigenic site, but does mean that the degraded form of enzyme still retains antigenic sites and that the binding of antibody to such sites affect the catalytic activity of enzyme significantly. In the following experiments, the effect of antibody on hexose-6-phosphate dehydrogenase activity was assayed with deoxyglucose 6-phosphate as substrate.

Effect of antibody on membrane-bound hexose-6-phosphate dehydrogenase

Incubation of intact microsomes with antibody did not affect the hexose-6-phosphate dehydrogenase activity at all, as shown in Fig. 4. It was thus concluded that none of the antigenic sites of this enzyme is exposed on the cytoplasmic side of the membrane. On the contrary, the ezyme was almost completely inhibited by antibody after treatment of microsomes with either deoxycholate or phospholipase A (Fig. 5). This implies that the enzyme molecules in deoxycholate- or phospholipase A-treated microsomes are able to make contact freely with antibody. In phospholipase C-treated microsomes, the hexose-6-phosphate dehydrogenase activity was only 50% inhibited.

Recently, Kreibich et al. [16] reported that the permeability of microsomal membranes can be modified by low concentrations of detergents without significantly altering membrane assembly, and that trypsin can be introduced into the luminal space of microsomes in the presence of 0.05% deoxycholate. It is thus possible to discriminate the membrane proteins either as the one located on the cytoplasmic surface or as the one located on the luminal surface, based

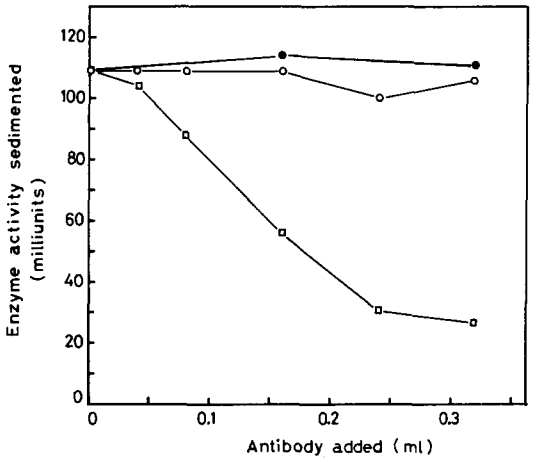

Fig. 4. Effect of antibody on the hexose-6-phosphate dehydrogenase activity of intact microsomes. Intact microsomes (14 mg protein) were incubated overnight at 0—4°C with the indicated amounts of antibody in 20 mM Tris buffer/0.25 M sucrose, pH 7.5. After centrifuging, the resulting pellets were washed with 20 mM Tris buffer/0.25 M sucrose, pH 7.5, with or without 1.0 M NaCl and suspended in a small volume of 0.25 M sucrose/0.5% deoxycholate. Hexose-6-phosphate dehydrogenase was assayed with NADP and deoxyglucose 6-phosphate as substrates, □———□, microsomes incubated with antibody and washed without NaCl; ○———○, microsomes incubated with antibody and washed with NaCl; ●———●, microsomes incubated with control γ-globulin and washed without NaCl. Data show that non-specific absorption of antibody on microsomes can be removed by 1.0 M NaCl.

on the findings of trypsin digestion in the presence or absence of deoxycholate. Using such techniques, Nilsson and Dallner [17] demonstrated the asymmetrical distribution of various microsomal enzymes.

Similar experiments on hexose-6-phosphate dehydrogenase yielded the

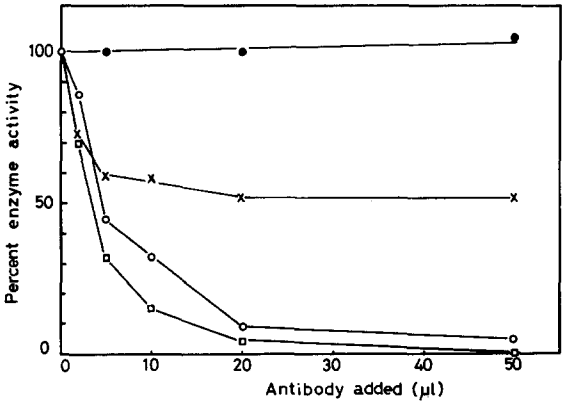

Fig. 5. Inhibition of microsomal hexose-6-phosphate dehydrogenase activity by antibody. Microsomes were treated with deoxycholate, phospholipase A or phospholipase C as described previously [3]. The treated microsomes were incubated with antibody for 20 min at 21°C in 50 mM Tris · HCl, pH 7.5, and then the hexose-6-phosphate dehydrogenase activity was assayed with NADP and deoxyglucose 6-phosphate as substrates. Deoxycholate-, phospholipase A- and phospholipase C-treated microsomes had a specific activity of 10.7, 11.2 and 4.5 munits per mg protein, respectively. ○———○, deoxycholate-treated microsome; □———□, phospholipase A-treated microsome; X———X, phospholipase C-treated microsome; ●———●, deoxycholate-treated microsome and control γ-globulin.

TABLE II

EFFECTS OF DEOXYCHOLATE AND/OR TRYPSIN ON MICROSOMAL ENZYMES

Microsomal pellets were suspended in 0.25 M sucrose at a protein concentration of 20 mg per ml. To 1.4-ml aliquots of the suspension were added 6.3 ml of 50 mM Tris buffer/50 mM KCl/0.25 M sucrose/0.033% deoxycholate, pH 7.5. The mixtures were incubated with or without trypsin (36 μg per mg micromsal protein) for 30 min at 30°C, and centrifuged at 144 000 × *g* for 40 min. The pellets were suspended in a small volume of 0.25 M sucrose and assayed for protein and enzyme activities. Hexose-6-phosphate dehydrogenase activity toward deoxyglucose 6-phosphate was measured at 21°C with the microsomes treated with 0.5% deoxycholate, and glucose-6-phosphatase activity was assayed at 30°C in the presence of 0.05% Triton X-100. Averages of two separate experiments with the same microsomal suspensions.

	Activities sedimented at 144 000 × *g* after treatments with			
	None	Deoxycholate	Trypsin	Deoxycholate + trypsin
Protein (mg)	17.9	14.8	12.1	10.3
Hexose-6-phosphate dehydrogenase (munits)	218	182	214	168
Glucose-6-phosphatase (μmol P_i released/min)	2.89	2.93	2.86	1.05
NADPH-cytochrome *c* reductase (μmol cytochrome *c* reduced/min)	0.72	0.70	0.05	0.07

results summarized in Table II, where effects of trypsin and/or deoxycholate on glucose-6-phosphatase and NADPH-cytochrome *c* reductase are also included for comparison. These enzymes are chosen as markers located on the luminal and cytoplasmic surface of the microsomal membrane [6,17—19], respectively. In agreement with the data of Nilsson and Dallner [17], neither solubilization nor inactivation of the marker enzymes occurred in the presence of 0.027% deoxycholate. In addition, trypsin inactivated glucose-6-phosphatase when deoxycholate was present. On the other hand, hexose-6-phosphate dehydrogenase was not affected in similar manner by the same treatments, i.e. hexose-6-phosphate dehydrogenase was solubilized slightly (15%), but definitely in the presence of 0.027% deoxycholate. Though not shown in Table II, 95% of the hexose-6-phosphate dehydrogenase activity was solubilized when the deoxycholate concentration was raised to 0.05%, at which concentration glucose-6-phosphatase was still bound to the membrane. Thus, hexose-6-phosphate dehydrogenase appears to be loosely associated with the membrane, like nucleoside diphosphatase or β-glucuronidase. In addition, the activity of hexose-6-phosphate dehydrogenase was lowered only 23% by the trypsin plus deoxycholate treatment and this change was not due to inactivation, but due to solubilization (20% of the activity was recovered from the washing of treated microsomes). At first sight this seemed to indicate that hexose-6-phosphate dehydrogenase would be embedded so deeply in the membrane that trypsin would fail to attack the enzyme even from the luminal side. However, taking into account that this enzyme retains full activity even after the molecular weight is reduced to 60% of the original as a result of tryptic digestion, the above data might not necessarily mean that trypsin failed to attack the enzyme. In order to clarify this point, the microsomes treated with trypsin and/or deoxycholate were electrophoresed on polyacrylamide gels. As a result, it was

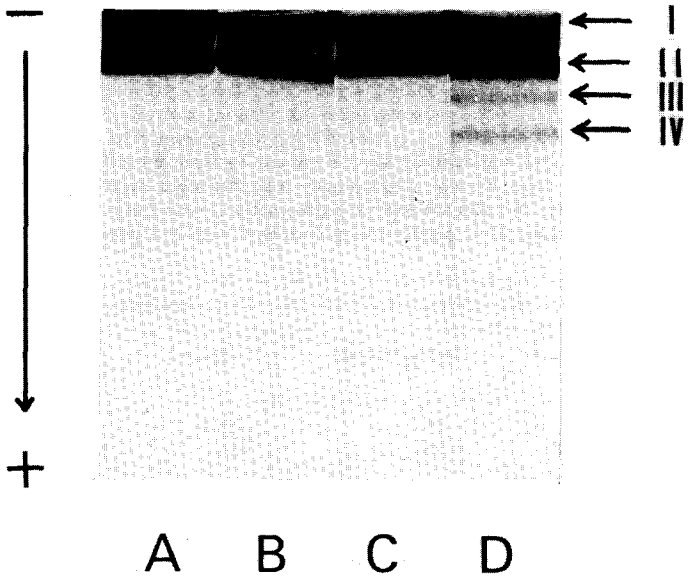

Fig. 6. Polyacrylamide disc gel electrophoresis of hexose-6-phosphate dehydrogenase. Microsomal suspensions were treated with deoxycholate and/or trypsin in the same condition as described in the legend of Table II. Suspended pellets of the treated microsomes were treated with 0.5% deoxycholate and then applied to electrophoretic columns. Electrophoresis and hexose-6-phosphate dehydrogenase staining were carried out as described previously [3]. Note that hexose-6-phosphate dehydrogenase of the microsomes pretreated with deoxycholate (B) or trypsin (C) as well as the enzyme of control microsomes (A) are intact (Bands I and II), while the enzyme in the microsomes pretreated with trypsin in the presence of deoxycholate (D) is degraded (Bands III and IV).

demonstrated that hexose-6-phosphate dehydrogenase was degraded by trypsin only when deoxycholate was present (Fig. 6). This suggests that the enzyme is located on the luminal side of the microsomal membrane.

TABLE III

EFFECTS OF DEOXYCHOLATE AND/OR ANTIBODY ON HEXOSE-6-PHOSPHATE DEHYDROGENASE ACTIVITY IN INTACT MICROSOMES

Intact microsomes, which contained 220 munits of hexose-6-phosphate dehydrogenase activity in a latent form, were incubated with antibody (0.13 mg protein) or control γ-globulin (1.0 mg protein) overnight at 0—4°C in 50 mM Tris buffer/50 mM KCl/0.25 M sucrose/0.027% deoxycholate, pH 7.5. The mixtures were centrifuged at 144 000 × *g* for 40 min, and the precipitate was washed once with 20 mM Tris buffer/0.25 M sucrose/1.0 M NaCl, pH 7.5. Hexose-6-phosphate dehydrogenase activity of the sediment was assayed with deoxyglucose 6-phosphate as substrate at 21°C after treatment with 0.5% deoxycholate. The results were averages of two separate experiments with the same microsomal suspensions.

Treatments	Enzyme activity sedimented (munits)
None	193
Control γ-globulin	193
Antibody	189
Deoxycholate	122
Deoxycholate + control γ-globulin	116
Deoxycholate + antibody	61

It has also been found that antibody becomes accessible to hexose-6-phosphate dehydrogenase if a low concentration of deoxycholate is present (Table III). This may be explained by one of the following alternatives: (a) subtle modifications of the membrane architecture by deoxycholate enable antibody to bind to hexose-6-phosphate dehydrogenase from the cytoplasmic surface; (b) antibody is introduced into the lumen so that it can bind to hexose-6-phosphate dehydrogenase from the luminal surface of the membrane. Some microsomal enzymes, such as nucleoside diphosphatase (molecular weight 100 000) and β-glucuronidase (molecular weight 290 000) are believed to be bound loosely to the luminal surface of the membrane, and are easily released from the membrane in the presence of deoxycholate [7,17,20,21]. Therefore, it seems not unreasonable to assume that antibody crossed the microsomal membrane in the presence of deoxycholate.

In short, the experiments with antibody suggested that hexose-6-phosphate dehydrogenase might be one of the enzymes which are bound loosely to the luminal side of microsomal membranes.

Permeability of membranes to substrates

The latency of a membrane-bound enzyme cannot be explained solely by its intramembraneous location. As a matter of fact, the enzyme would be fully active regardless of its intramembraneous location as long as the substrate penetrates freely into the membrane. In the case of hexose-6-phosphate dehydrogenase, it is therefore reasonable to assume that either glucose-6-phosphate or NADP or both are unable to penetrate the membrane. In order to examine which substrate may be primarily responsible for the hexose-6-phosphate

TABLE IV

EFFECTS OF TEMPERATURE AND SUCROSE ON THE LATENCY OF MICROSOMAL ENZYME ACTIVITIES

Enzyme activities of the microsomal suspensions were assayed with or without 0.25 M sucrose at 21°C or 37°C. Total activities of hexose-6-phosphate dehydrogenase and β-glucuronidase were assayed with the microsomes treated with 1.0% and 0.4% Triton X-100, respectively. Total activities of glucose-6-phosphatase and nucleoside diphosphatase were assayed in the presence of 0.05% and 0.02% Triton X-100, respectively. The results are expressed as: hexose-6-phosphate dehydrogenase, munits per mg protein; glucose-6-phosphatase and nucleoside diphosphatase, μmol P_i released per min per mg protein; and β-glucuronidase, nmol phenolphthalein released per min per mg protein. Averages of three experiments.

	21°C			37°C		
	None	Sucrose	Triton	None	Sucrose	Triton
Hexose-6-phosphate dehydrogenase						
Glucose 6-phsophate	0	0	3.59	1.29	0	8.31
Deoxyglucose 6-phosaphte	0	0	10.88	1.23	0.21	29.72
Galactose 6-phosphate	0	0	2.66	0.30	0	6.72
Glucose-6-phosphatase						
Glucose 6-phosphate	0.078	0.077	0.155	0.285	0.290	0.309
Deoxyglucose 6-phosphate	0.014	0.009	0.131	0.070	0.065	0.229
Galactose 6-phosphate	0.010	0.010	0.050	0.057	0.059	0.068
Nucleoside diphosphatase	0.188	0.137	0.844	0.312	0.258	1.047
β-Glucuronidase	2.57	1.85	2.39	8.07	5.66	7.93

dehydrogenase latency, some experiments were performed (Table IV) and the results are summarized as follows:

(1) At 21°C, intact microsomes show no hexose-6-phosphate dehydrogenase activity, but show about 50% of the total glucose-6-phosphatase activity. This suggests that the latency of hexose-6-phosphate dehydrogenase is not due primarily to the impermeability of membranes to glucose 6-phosphate.

(2) At 37°C, microsomes come to show approx. 16% of the total hexose-6-phosphate dehydrogenase activity (glucose 6-phosphate as substrate), while their glucose-6-phosphatase activity is revealed as much as 92%. This indicates that glucose 6-phosphate can penetrate almost freely into the membrane at this temperature, and that the membrane becomes also permeable to NADP, though to a limited extent.

(3) Among the three substrates tested, glucose 6-phosphate appears to penetrate the membrane most rapidly and deoxyglucose 6-phosphate does so most slowly. This inference is based on the comparison of the activity of glucose-6-phosphatase or hexose-6-phosphate dehydrogenase in the presence and absence of Triton X-100, i.e. in the case of glucose-6-phosphatase, the activities against glucose 6-phosphate, deoxyglucose 6-phosphate and galactose 6-phosphate at 37°C in the absence of Triton X-100 were 92.2%, 30.6% and 83.8% of the activities in the presence of Triton X-100, respectively. The same holds for the hexose-6-phosphate dehydrogenase activity.

(4) Unexpectedly, sucrose was inhibitory to the activities of hexose-6-phosphate dehydrogenase, nucleoside diphosphatase and β-glucuronidase to varying degrees, but not to the glucose-6-phosphatase activity. Since sucrose has no inhibitory effect on purified hexose-6-phosphate dehydrogenase (and probably so on the other enzymes), the effect observed might be interpreted in terms of the modification of membrane permeability to NADP, IDP and phenolphthalein glucuronide by sucrose. Isotonic KCl was without effect on the activities of the four enzymes at both temperatures tested.

In an additional experiment, the microsomes were incubated with NADP plus deoxyglucose 6-phosphate or NADP alone at 37°C for 30 min, washed twice with 20 mM Tris buffer, pH 7.5, sonicated in a small volume of the buffer and assayed for NADPH. The results showed that no detectable amount of NADPH was accumulated within the luminal space of the microsomes. This rules out the possibility that the latency of hexose-6-phosphate dehydrogenase might be due to the accumulation of reaction products within the luminal space.

In short, glucose 6-phosphate appears to penetrate the microsomal membrane almost freely at 37°C, while NADP does so only slowly. This would be the primary cause of the hexose-6-phosphate dehydrogenase latency.

Effect of sucrose on membrane hydrophobicity

Based on assumptions that sucrose-induced changes in the membrane permeability to enzyme substrates might be due to the changes in the hydrophobicity of membranes, and that such changes in hydrophobicity might affect the ANS fluorescence of microsomes, effects of sucrose on the ANS fluorescence of microsomes as well as that of micellar phospholipids were examined.

Intact microsomes as well as phosphatidylcholine enhanced greatly the

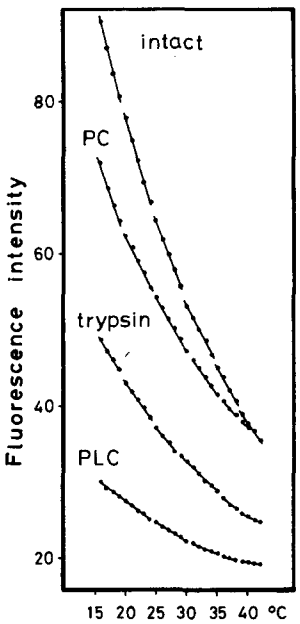

Fig. 7. Effect of temperature on the ANS fluorescence of microsomes and phospholipid. Microsomes were treated with phospholipase C (33 μg per mg microsomal protein at 37°C for 30 min) or trypsin (27 μg per mg microsomal protein at 37°C for 60 min). Phospholipid micelles were prepared by sonicating 2 mg phosphatidylcholine in 1 ml 5 mM Tris buffer, pH 7.6. Intact or treated microsomes (0.3 mg microsomal protein) or phospholipid (0.1 mg) were added to 3 ml of the medium and ANS fluorescence was measured as described in Materials and Methods. Intact, untreated microsomes; PLC, phospholipase C-treated microsomes; trypsin, trypsin-treated microsomes; PC, phosphatidylcholine micelles.

fluorescence of ANS, while phosphatidylethanolamine was much less effective in this respect; i.e. the fluorescence intensity with phosphatidylethanolamine was approx. 1/30 that with phosphatidylcholine.

The magnitude of fluorescence enhancement was greater with fresh microsomes than with microsomes stored at 4°C or −70°C.

Effects of temperature on the ANS fluorescence of microsomes and phosphatidylcholine are shown in Fig. 7. As is evident in this figure, the ANS fluorescence of intact microsomes shows breaks at 19, 25, 30, 35 and 39°C. Breaks at the same temperatures are also evident with phosphatidylcholine, while the fluorescence of phosphatidylethanolamine showed no temperature-dependent changes (not illustrated).

Treatment of microsomes with phospholipase C resulted in a drastic decrease of the ANS fluorescence, but had little effect on the temperatures at which breaks occurred, though the breaks became less prominent.

Trypsin treatment also suppressed the ANS fluorescence of microsomes, but a similar suppression was also noted with the microsomes treated in the same way, but without trypsin. Accordingly, the difference between intact and trypsin-treated microsomes would not be attributed to trypsin-digestable proteins of the microsomes.

Addition of sucrose to the reaction mixture was without effect on the ANS fluorescence of intact microsomes and phosphatidylcholine. The breaks occurred at the same temperatures in the presence as in the absence of sucrose.

Hexose-6-phosphate dehydrogenase also enhanced the ANS fluorescence to a measurable extent. The fluorescence was, however, not dependent on temperature and was not affected by sucrose.

In conclusion, an attempt to demonstrate sucrose-induced changes in the hydrophobicity of microsomal membranes by means of ANS fluorescence was fruitless.

Discussion

In the previous report [3], we assumed that hexose-6-phosphate dehydrogenase is one of the most firmly bound enzymes among microsomal proteins. This assumption is based upon the findings that hexose-6-phosphate dehydrogenase would not be released from microsomes by washing with 1 M NaCl and by treatments with lipid hydrolases and trypsin. The present experiments demonstrated, however, that hexose-6-phosphate dehydrogenase is actually easily solubilized by deoxycholate at a lower concentration than that required for solubilizing other membrane-bound enzymes, such as glucose-6-phosphatase and UDPglucuronyltransferase (EC 2.4.1.17). Thus, it became clear that the enzyme is loosely bound to the luminal surface of the microsomal membranes.

The highly purified hexose-6-phosphate dehydrogenase does not require the presence of lipids for activity and does not form aggregates in the absence of detergents. As well as its loose binding to the membrane, these properties suggest that the enzyme is rather hydrophilic as a whole, although it does possess hydrophobic portions as evidenced by its ability to increase ANS fluorescence. The hydrophobic region that probably plays a role in binding the enzyme to the membrane may not be removed from the enzyme by trypsin digestion, since the catalytically active region of hexose-6-phosphate dehydrogenase is still bound to the membrane even after the treatment of microsomes with phospholipase C and trypsin [3]. This is in contrast to the case of cytochrome b_5 which may be separated into a catalytically active portion and hydrophobic anchor portion by trypsin [22—25].

The present findings that hexose-6-phosphate dehydrgenase is loosely bound on the luminal surface of the microsomal membrane do not explain the latency of the enzyme. If the membrane is permeable to glucose 6-phosphate and NADP, then the enzyme would be fully active regardless of its intramembraneous location. It is, therefore, reasonable to assume that either glucose 6-phosphate or NADP or both do not freely penetrate the membrane, which is semipermeable [26—28].

Recent studies by Nilsson et al. [29] indicated that uncharged substances of molecular weight of up to at least 600 freely penetrate into the membrane, but charged substances such as acetate, mevalonate and glucose 6-phosphate do not. Due to some experimental difficulty, they failed to obtain reliable data on the permeability of IDP, IMP, NAD and other substances, but it was inferred that these substances as well as UDPglucuronic acid may penetrate the membrane only slowly.

The present data demonstrated, however, that glucose 6-phosphate penetrates the membrane almost freely at 37°C, while IDP and NADP do so very slowly. This indicates that the latency of hexose-6-phosphate dehydrogenase is

primarily due to the impermeability of the microsomal membrane to NADP.

The activity of β-glucuronidase was at the same level in the presence and absence of Triton X-100 (Table IV). This does not mean, however, that phenolphthalein glucuronide penetrates the membrane freely, since the activity was measured at pH 4.5, at which pH microsomes were sedimented and hardly regarded as intact.

The present findings also indicate that although effects of sucrose on membrane permeability vary with various substances, sucrose is by no means an adequate substance to maintenance of isotonicity in the study of membrane permeability.

ANS has been used as a probe for the apolar regions of proteins and also as a conformational probe when the conformational change involves exposure of hydrophobic groups [30—36]. On the other hand, ANS fluorescence of biological membranes is attributable largely to the phospholipid moieties [37,38] which undergo temperature-dependent phase transitions and have a great influence on the activities of membrane-bound enzymes [39—47]. It seems probable, therefore, that suppression of hexose-6-phosphate dehydrogenase activity by sucrose might be the result of hydrophobicity (or fluidity) changes of membrane lipids, and that if so, such effect of sucrose on lipids could be manifested by comparing the ANS-microsome fluorescence in the presence and absence of sucrose. Such possibility was, however, ruled out in the present experiments. The mechanism of sucrose effects on membrane permeability thus remains to be explored.

Acknowledgement

This work was supported by grant 154255 from the Ministry of Education.

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