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SOLUBLE AND MICROSOMAL FORMS OF NADH-CYTOCHROME b_5 REDUCTASE FROM HUMAN PLACENTA

SIMILARITY WITH NADH-METHEMOGLOBIN REDUCTASE FROM HUMAN ERYTHROCYTES

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Summary

In congenital methemoglobinemia associated with mental retardation a generalized deficiency of NADH-cytochrome b_5 reductase (NADH : ferricytochrome b_5 oxidoreductase, EC 1.6.2.2) has been found in soluble extracts of red blood cells, as well as in deoxycholate-treated extracts of leukocytes, muscle, liver and fibroblasts (Leroux et al. (1975) Nature 258, 619—620). In the present study the relationship between the microsomal (I) and the soluble (II) NADH-cytochrome b_5 reductase was investigated, using human placenta as a source of enzyme. Both forms were compared to the human red-cell soluble NADH-methemoglobin reductase (III) and NADH-cytochrome b_5 reductase (IV). The four entities exhibited great immunological similarities. It is concluded that the three soluble enzymes (II, III and IV) are identical. The detergent-solubilized microsomal NADH-cytochrome b_5 reductase (I) is immunologically very similar to the soluble enzymes, but presents distinct features possibly due to the presence of a hydrophobic part.

Introduction

In the red blood cells of patients suffering from congenital recessive methemoglobinemia, a concomitant defect of both NADH-methemoglobin reductase (or NADH-diaphorase) and NADH-cytochrome b_5 reductase (NADH : ferricytochrome b_5 oxidoreductase, EC 1.6.2.2) has been found [1,2,3]. Moreover in cases of methemoglobinemia associated with mental retardation, both enzyme activities are missing, not only in soluble extracts of red blood cells,

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but also in deoxycholate-treated extracts of leukocytes, muscle, liver and fibroblasts [4], suggesting that this condition is due to a generalized deficiency of both soluble and microsomal NADH-cytochrome b_5 reductase. On these genetic grounds the following assumptions can be made: (i) red-cell NADH-methemoglobin reductase is actually a soluble form of NADH-cytochrome b_5 reductase; (ii) soluble and microsomal NADH-cytochrome b_5 reductase are closely related or even identical entities. The finding by Hultquist and Passon [5] that cytochrome b_5 plays a role in methemoglobin reduction, and the identification by the same authors of a soluble cytochrome b_5 reductase in red blood cells [6] lends further support to point (i).

In the present study we bring evidence that hypotheses (i) and (ii) are valid in showing that human red-cell NADH-methemoglobin reductase is immunologically identical to both soluble and microsomal forms of NADH-cytochrome b_5 reductase isolated from human placenta.

Material and Methods

Chemical and reagents

Reduced nicotinamide adenine dinucleotide (NADH), 2,6-dichlorophenol-indophenol, 3-(4,5 dimethyl thiazolyl-2)-2,5 diphenyl tetrazolium bromide (MTT) and Triton X-100 were obtained from Sigma. DEAE-Sephadex A 50, DEAE-cellulose 52 and Sephadex G-100 were purchased from Sephadex and Whatman. BioGel A 0.5 was from BioRad. Other chemicals were reagent grades from different commercial sources.

Trypsin-solubilized cytochrome b_5

Cytochrome b_5 was purified to homogeneity from rat liver microsomes according to the procedure of Omura and Takesue [7] in which trypsin is used to extract the protein.

Enzyme assays

All assays were carried out at 25°C.

(a) NADH methemoglobin-ferrocyanide reductase activity was assayed according to the method of Hegesh et al. [8] in a 1-ml reaction mixture containing 5 μmol of citrate buffer pH 4.7, 0.5 μmol EDTA, 0.1 μmol potassium ferricyanide, 18 nmol of semi-purified hemoglobin and 0.15 μmol NADH. This method is based on the fact that the methemoglobin-ferrocyanide complex is a more efficient substrate in vitro than native methemoglobin [9].

(b) NADH-diaphorase activity was measured according to the method of Scott and McGraw [10] in a 1-ml reaction mixture containing 32 μmol of Tris buffer pH 7.5, 1 μmol EDTA, 0.1 μmol of dichlorophenol-indophenol and 0.1 μmol NADH.

(c) NADH-ferricyanide reductase activity was assayed according to the method of Zamudio and Canessa [11] in a 1 ml reaction mixture containing 240 μmol of Tris buffer pH 8.5, 0.5 μmol potassium ferricyanide and 0.2 μmol NADH.

In each enzyme assay the reaction was started by addition of NADH, and the reduction of methemoglobin-ferrocyanide complex, dichlorophenol-indophenol

and ferricyanide were followed by recording the absorbance change at 578 nm, 600 nm and 420 nm respectively. Values of 42.0 $mM^{-1} \cdot cm^{-1}$ [8], 21.0 $mM^{-1} \cdot cm^{-1}$ [12] and 0.96 $mM^{-1} \cdot cm^{-1}$ [11] were used for the molar extinction coefficient of methemoglobin-ferrocyanide complex, dichlorophenol-indophenol and ferricyanide, respectively.

The enzyme units were expressed as nmol of substrate reduced per min for the methemoglobin-ferrocyanide reductase [8], and as μmol of dichlorophenol-indophenol or ferricyanide reduced per min for the two other enzyme assays [10,11].

(d) NADH-cytochrome b_5 reductase activity was assayed according to the method of Mihara and Sato [12], in a 0.5-ml reaction mixture containing 50 μmol of potassium phosphate buffer pH 7.5, and 0.005 μmol of cytochrome b_5. The reaction was started by addition of 0.05 μmol of NADH and followed at 556 nm on a Gilford 2400 spectrophotometer with a full scale expansion of 0.1 absorbance unit and rapid chart speed (2 inch/min). Under these conditions, the blank without enzyme was nul and the enzymatic reaction was pseudo-first order with respect to cytochrome b_5 concentration. The velocity was defined as the apparent first order reaction constant, k (min^{-1}). Results are expressed as $k \cdot mg^{-1}$ proteins.

Polyacrylamide disc gel electrophoresis

This was performed in the system of Ornstein [13] using a 7.5% migration gel and a 2.5% stacking gel. The electrophoresis was performed at 4°C with a constant current of 3 mA/tube. The cathode chamber contained a 0.05 M Tris/glycine buffer pH 9.2 and the anode chamber contained a 0.1 M Tris · HCl buffer pH 9.2. The gels were stained for protein in a solution of 0.25% Coomassie Brilliant Blue. The gels were also stained for enzymatic (NADH-diaphorase) activity according to the dichlorophenol-indophenol-MTT linked method of Kaplan and Beutler [14]. The reagent mixture contained 1.2 mM MMT, 0.06 mM dischlorophenol-indophenol and 1.3 mM NADH in a 0.25 M Tris · HCl buffer pH 8.4.

Electrofocusing

This was performed in acrylamide gel according to the method of Drysdale et al. [15] using a 7.5% acrylamide gel and 2% Ampholine carrier ampholytes with a pH range between 3.5 and 10. The gels were stained for enzymatic activity in the solution described above.

To each gel 5 μl of hemoglobin solution (12 mg/ml) devoid of diaphorase activity was added as a colored marker allowing an exact comparison between the different gels.

Other methods

Hemoglobin concentration was measured according to the method of Zijlstra and Von Kampen [16]. The protein concentration was determined by the method of Lowry et al. [17] using bovine serum albumin as a standard. Chicken anti-human red cell methemoglobin reductase was prepared as described previously [18].

Results

I. Preparation of enzymes

Purification of human red cell NADH-methemoglobin reductase. NADH-methemoglobin reductase was purified from human red cells with a procedure derived from that described by West [19]. Outdated, washed, packed red cells (500 ml) were lyzed by adding an equal volume of 1 mM EDTA containing toluene (50 ml/l). After shaking for 10 min, the lyzate was centrifuged for 40 min at 3000 × *g*, the upper toluene and lipid layers removed, and the clear middle portion decanted. This fraction was diluted with 2 vols. of 5 mM potassium phosphate buffer pH 7 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 per ml of packed red cells. The resin was extensively washed on a Buchner funnel with the buffer until the effluent was colourless. The enzyme was eluted with 50 mM potassium phosphate buffer pH 5.8 containing 0.1 mM EDTA and 0.3 M KCl, and precipitated by $(NH_4)_2SO_4$ added to 60% saturation. The precipitate was removed by centrifugation, suspended in a minimum volume of 5 mM phosphate buffer, pH 6.5, containing 0.1 M EDTA and 0.05 M KCl and dialyzed against this medium overnight at 4°C. The dialyzed fraction was applied to a 1.5 × 14-cm DEAE-Sephadex A 50 column equilibrated with the phosphate/EDTA/KCl buffer pH 6.5. The column was washed with this buffer at a flow rate of 25 ml/h, and the peak of enzyme activity emerged immediately after a

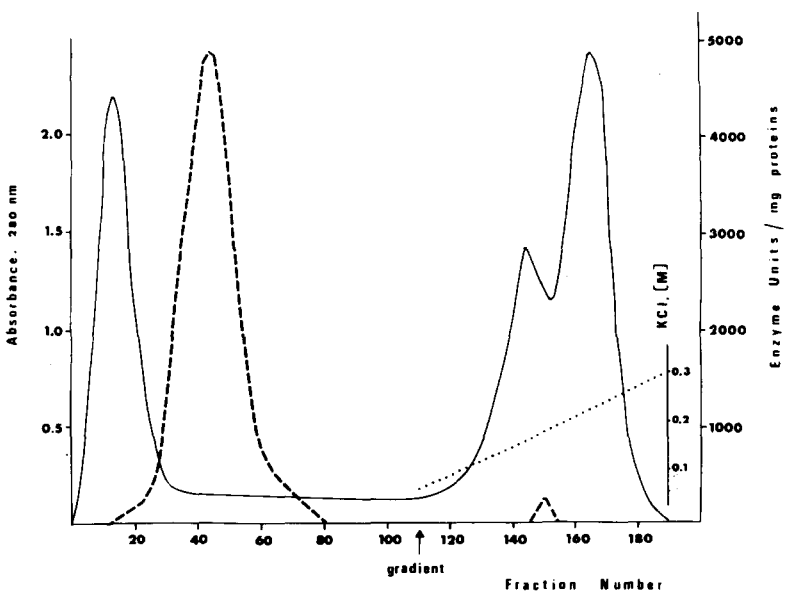

Fig. 1. Typical DEAE-Sephadex A 50 chromatographic pattern of human red cell methemoglobin reductase. ———, protein (absorbance at 280 nm); - - - - - -, NADH-methemoglobin reductase activity expressed as nmol of methemoglobin-ferrocyanide complex [8] reduced · min^{-1} · mg^{-1} proteins. Experimental procedure described under Results. It should be noted that practically no enzyme activity was recovered when a linear salt concentration gradient was applied after the main peak of enzyme had been spontaneously eluted.

TABLE I

PURIFICATION OF HUMAN ERYTHROCYTE METHEMOGLOBIN REDUCTASE

Fraction	Total protein (mg)	Total activity (units)	Specific activity (units */mg)	Yield (%)
Hemolysate	63 750	153 000	2.4	100
DEAE-cellulose 52 eluate	400	109 200	273	72
Dialysed $(NH_4)_2SO_4$ fraction	120	108 428	906	71
DEAE-Sephadex A 50 (concentrated eluate)	25.2	97 666	3876	64

* The enzyme activity was estimated according to the methemoglobin-ferrocyanide method of Hegesh et al. [8]. Units are expressed as nmol of substrate reduced per min.

major peak of unadsorbed proteins. The enzyme was loosely bound to the resin and it was eluted without need of increasing the ionic strength (Fig. 1). The enzyme fraction was concentrated twently fold by ultrafiltration on Diaflo membrane UM 10. The purification of NADH-methemoglobin reductase is summarized in Table I. The purest fractions have a specific activity of about 4000 units per mg of protein. The preparation was stable for several months when stored at −20°C. Only slight contamination by other proteins devoid of NADH-diaphorase activity was found after disc gel electrophoresis. This preparayion was injected to chicken to prepare anti-methemogobin reductase antiserum [18].

Purification of human red-cell NADH-cytochrome b_5 reductase. NADH-cytochrome b_5 reductase was purified from human erythrocytes according to the method of Passon and Hultquist [6]. During the purification the enzyme was monitored by its NADH-ferricyanide reducing activity [11]. In the final preparation the NADH-cytochrome b_5 reductase activity was 80 $min^{-1} \cdot mg^{-1}$ protein using cytochrome b_5 as electron acceptor [12].

Purification of human placenta soluble and microsomal NADH-cytochrome b_5 reductase. (a) Preparation of placental extracts. Human term placentae were obtained within 2 h after delivery from the Maternity Hospitals Port-Royal and Baudelocque. After removal of membranes and vessels the placental villi were extensively washed with 0.15 M KCl. The tissue was minced and thoroughly washed with 10 l of 0.15 M KCl and 5 l of 5 mM phosphate buffer pH 7.2, until the washings became almost colourless. The washed placental tissue was homogenized in a Waring blendor with 3 vols. of 0.25 M sucrose in 5 mM phosphate buffer pH 7.2. All the experiments were carried out at +4°C. The homogenate was centrifuged twice at 30 000 × *g* for 30 min and the pellet discarded. Microsomes were then sedimented by centrifugation at 105 000 × *g* for 90 min. Both the supernatant cytosolic fraction and the pellet of microsomes were collected. The method yielded about 1 g microsomal protein from 2500 g of placental tissue.

(b) Purification of cytosolic NADH-cytochrome b_5 reductase. The supernatant obtained from 600 g placental tissue after centrifugation at 105 000 × *g* for 90 min was used for purification of the enzyme. In this fraction the contamination by erythrocyte methemoglobin reductase, estimated from the amount of contaminating hemoglobin, did not exceed 3—5% of the total

enzyme activity. The preparation was adjusted to pH 7.2 with KOH and loaded at a rate of 150 ml/h on to a 2.4 × 20-cm DEAE-cellulose 52 column previously equilibrated with 1 mM phosphate buffer pH 7.2. The elution and subsequent steps were exactly the same as in the purification protocol of NADH-cytochrome b_5 reductase from red cells [6]. The final activity of NADH-cytochrome b_5 reductase was 12.7 $min^{-1} \cdot mg^{-1}$ protein, using cytochrome b_5 as electron acceptor [12].

(c) Preparation of microsomal NADH-cytochrome b_5 reductase. The microsomal pellet, prepared as described above, was washed once with 0.15 M KCl and once with 0.1 M potassium phosphate buffer pH 7.5, and then suspended in 20 mM Tris · HCl buffer pH 8.0. In order to obtain the intact form of the enzyme, the method described by Mihara and Sato [12] was selected. According to this method the cytochrome b_5 reductase was solubilized by a mixture of 1% Triton X-100 and 1% sodium deoxycholate. The detergent solubilized cytochrome b_5 reductase was obtained after ammonium sulphate fractionation, followed by three chromatographic steps on DEAE-Sephadex A 50 [12]. All steps were performed in the presence of 0.5% Triton X-100. The final NADH-cytochrome b_5 reductase activity was 20 $min^{-1} \cdot mg^{-1}$ protein using cytochrome b_5 as electron acceptor [12].

The purity of both soluble and microsomal cytochrome b_5 reductase was controlled by disc electrophoresis in 7.5% polyacrylamide gel at pH 9.2 which showed some contamination by other proteins. However, for the purpose of this work it was not imperative to achieve complete purification of each enzyme, since all electrophoretic and immunologic techniques which were subsequently applied involved selective and specific staining and assays.

II. Comparison between the red-cell and placental enzymes

(a) Molecular weight estimation. Upon gel filtration through Biogel A 0.5, the three soluble enzymes (red-cell NADH-methemoglobin reductase and NADH-cytochrome b_5 reductase, and placental cytosolic NADH-cytochrome b_5 reductase) displayed an identical elution pattern corresponding to a mol. wt. of about 30 000.

(b) Electrophoretic studies. (i) Electrophoresis in polyacrylamide gel. Electrophoresis in polyacrylamide gel followed by specific staining for NADH-diaphorase activity showed two distinct bands (bands A and B) (Fig. 2) with identical mobility for the three soluble enzymes: erythrocyte NADH-methemoglobin reductase, erythrocyte NADH-cytochrome b_5 reductase and placental soluble NADH-cytochrome b_5 reductase. In the preparation of soluble cytochrome b_5 reductase from placenta an additional band was observed (band C) (Fig. 2). The microsomal cytochrome b_5 reductase from placenta exhibited a single slow-moving band (band D) (Fig. 2).

It is noteworthy that in fresh hemolysate the red cell NADH-methemoglobin reductase and cytochrome b_5 reductase showed a single band (band B) (Fig. 2). In the course of the purification procedures additional more anodic bands with diaphorase activity appear (Fig. 2). They clearly represent secondary enzyme deriving from the primary enzyme (band B).

(ii) Isoelectric focusing in polyacrylamide gel. Upon isoelectric focusing in a pH 3.5—10 gradient [15], three bands (I, II and III) (Fig. 3) with isoelectric

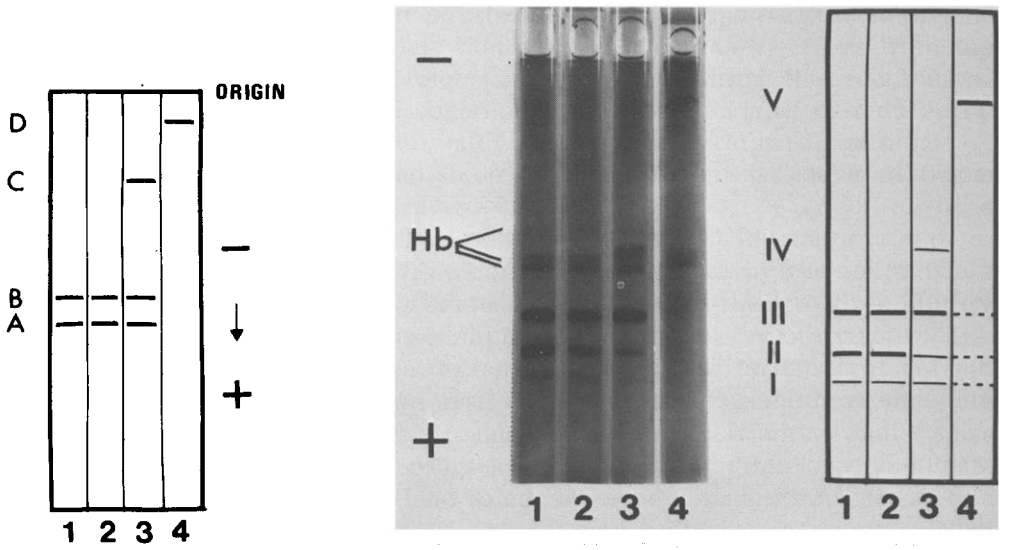

Fig. 2. Disc polyacrylamide gel electrophoresis of the four enzyme preparations. The enzyme was stained as NADH-diaphorase [14]. 1, Purified human red cell NADH-methemoglobin reductase. 2, Purified human red cell NADH-cytochrome b_5 reductase. 3, Semi-purified soluble cytochrome b_5 reductase from human placenta. 4, Semi-purified detergent solubilized microsomal cytochrome b_5 reductase from human placenta. The electrophoresis was performed according to the method of Ornstein [13].

Fig. 3. Electrofocusing in polyacrylamide gel (pH 3.5—10) according to the method of Drysdale et al. [15]. The enzymes were stained as NADH-diaphorase [14]. In the diagram the hemoglobin (Hb) bands seen on the photographs have been omitted, and only the enzyme bands indicated. The isoelectric pH are respectively 6.3 (band I), 6.5 (band II), 6.7 (band III), 7.15 (band IV), 8.0 (band V). 1, Purified human red cell NADH-methemoglobin reductase. 2, Purified human red cell NADH-cytochrome b_5 reductase. 3, Semi-purified soluble NADH-cytochrome b_5 reductase from human placenta. 4, Semi-purified detergent-solubilized microsomal NADH-cytochrome b_5 reductase from human placenta.

pH of 6.3, 6.5 and 6.7 respectively, were found for the two erythrocytic enzymes and for the soluble enzyme from placenta. Traces of enzyme with identical isoelectric pH were also detected for the microsomal enzyme. Two additional bands were observed for the two enzymes from human placenta: band IV with isoelectric pH 7.15 for the soluble enzyme, and band V with isoelectric pH about 8.0 for the microsomal enzyme (Fig. 3).

(c) Immunochemical studies. (i) Inactivation test by anti-human red cell methemoglobin reductase antiserum. Increasing amounts of antiserum were added to constant amounts (expressed as units of methemoglobin-ferrocyanide reducing activity) of the four enzymes studied. After incubation for 30 min at 37°C, followed by 12 h at +4°C, and centrifugation, the residual activity was measured. The inactivation curve obtained with the human erythrocytic NADH-methemoglobin reductase, erythrocytic cytochrome b_5 reductase and the soluble cytochrome b_5 reductase from placenta was identical (Fig. 4). With the last named enzyme however a biphasic curve was seen indicating the presence of an additional antigen with lesser affinity for the antiserum. With the preparation of microsomal cytochrome b_5 reductase a single slope was obtained, slightly different from that observed with the soluble entities (Fig. 4). In order to verify whether the nature and size of the substrate used to test the

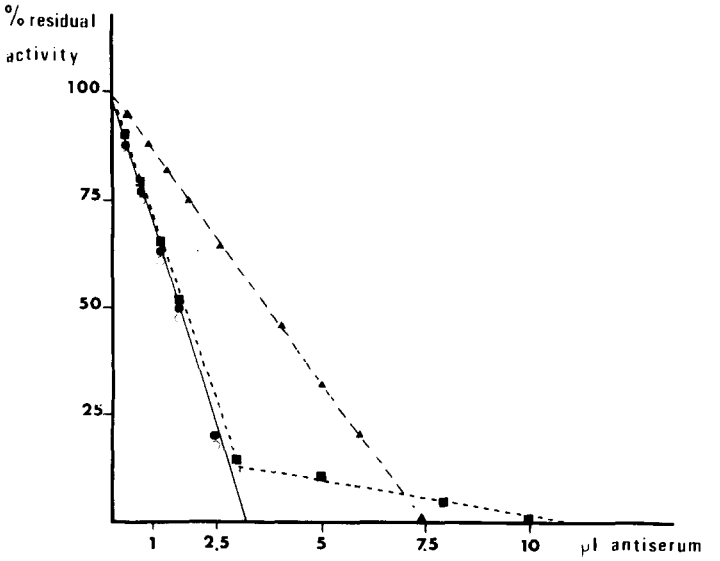

Fig. 4. Inactivation of the four enzymes by chicken antiserum prepared against human red cell methemoglobin reductase. The incubation mixture (60 µl) contained 5 µl of enzyme preparation, 10 µl of different dilutions of chicken antiserum and 45 µl of saline. Baseline activities of studied enzymes were made equal before incubation with antiserum. After incubation for 30 min at 37°C and 12 h at +4°C, whole volume (60 µl) of mixture was assayed for residual methemoglobin reductase activity according to the method of Hegesh et al. [8]. The results are expressed as percentage of the activity measured in control experiments in which antiserum was replaced by normal chicken serum. ○———○, human red cell methemoglobin reductase. ●———●, human red cell cytochrome b_5 reductase. ■- - - - -■, soluble cytochrome b_5 reductase from human placenta. ▲- - - - -▲, detergent-solubilized microsomal cytochrome b_5 reductase from human placenta.

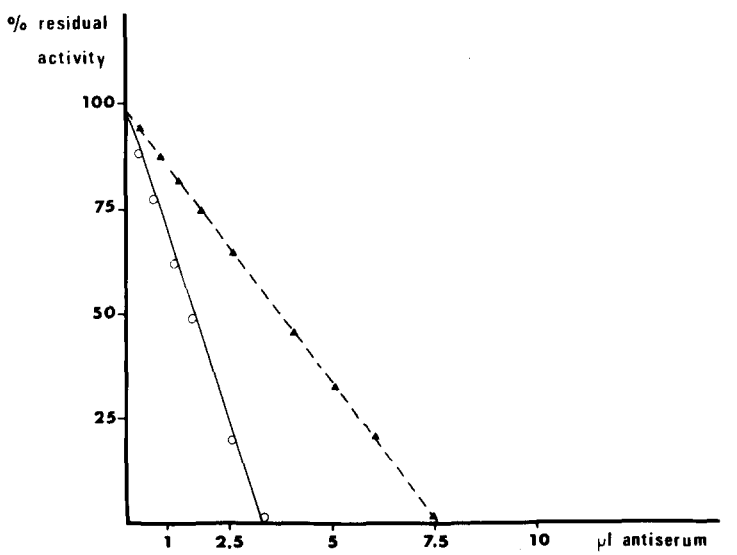

Fig. 5. Inactivation of human red cell methemoglobin reductase (○———○) and microsomal cytochrome b_5 reductase from human placenta (▲- - - - -▲) by chicken antiserum anti-human red cell methemoglobin reductase. Experimental procedure was as described in the legend to Fig. 4. The residual enzyme activity was measured for both enzymes as NADH-diaphorase [10], NADH-ferricyanide reductase [11], cytochrome b_5 reductase [12] and methemoglobin-ferrocyanide reductase [8]. Identical curves were obtained with the four different assays for each enzyme. Baseline activities of both enzymes were made equal for each enzyme assay before incubation with chicken antiserum. The results are expressed as percentage of the activity measured in control experiments in which antiserum was replaced by normal chicken serum.

residual activity could affect the slope of the inactivation curve, we have repeated the same experiment comparing four substrates of different mol. wts.: methemoglobin-ferrocyanide complex (65 000), t-cytochrome b_5 (12 000), dichlorophenol-indophenol (360) and potassium ferricyanide (324). The results indicate that the inactivation curve is identical with the four substrates (Fig. 5). Thus, the slight difference observed between the red cell methemoglobin reductase on one hand, and the microsomal cytochrome b_5 reductase on the other hand, remains unchanged, regardless of the kind of substrate used for determining the residual activity after incubation with the antiserum. It is therefore likely that the different slope obtained with the microsomal cytochrome b_5 reductase results from a different accessibility of the anti-methemoglobin reductase antiserum.

(ii) Determination of equivalence point. To increasing amounts of investigated enzyme a fixed amount of antiserum was added. After incubation for 30 min at 37° C and 60 min at +4° C, the residual activity was measured. A graphic construction enables to determine the amount of antiserum for which unbound enzymatic activity appears ('equivalence point'). This amount was identical for the three soluble enzymes, while the equivalence point was found to be slightly different for the microsomal enzyme (Fig. 6).

(iii) Immuniprecipitation was performed according to Ouchterlony [20]. The central well contained chicken antiserum directed against human red-cell

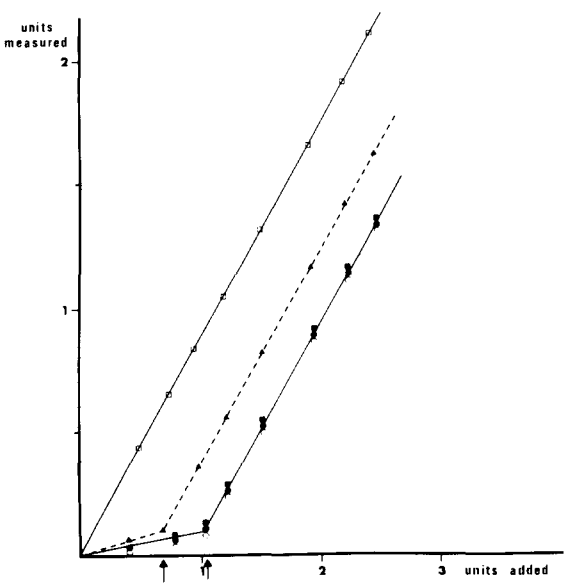

Fig. 6. Determination of the equivalence point of the four enzyme preparations with chicken antiserum directed against human red cells hemoglobin reductase. ○———○, human red cell NADH-methemoglobin reductase. ●———●, human red cell cytochrome b_5 reductase. ■———■, soluble cytochrome b_5 reductase from human placenta. ▲- - - - -▲, detergent-solubilized microsomal cytochrome b_5 reductase from human placenta. □———□, control with non-immunized chicken serum. Increasing amounts of enzyme preparations (0.5—16 units) were added to a constant volume of chicken antiserum. After incubation for 30 min at 37°C and 60 min at +4°C, the total amount of incubated mixture was assayed for residual methemoglobin-ferrocyanide reductase activity according to Hegesh [8]. The arrows indicate the equivalence points.

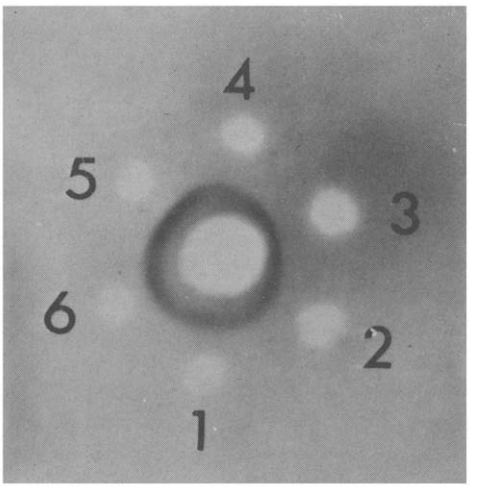

Fig. 7. Double immunodiffusion of the four enzyme preparations against anti-red cell methemoglobin reductase. Undiluted chicken antiserum was placed in the center well. In the surrounding wells were placed: 1 and 5, human red cell methemoglobin reductase; 2 and 6, detergent-solubilized microsomal cytochrome b_5 reductase from human placenta; 3, human red cell cytochrome b_5 reductase; 4, soluble cytochrome b_5 reductase from human placenta. The experiment was carried out in 2% agar, in 0.05 M veronal buffer, pH 8.6, 1 mM EDTA and 2% NaCl. The precipitation bands were allowed to develop in a humidity chamber at +4°C. After extensive washing, the plate was stained for NADH-diaphorase activity [14].

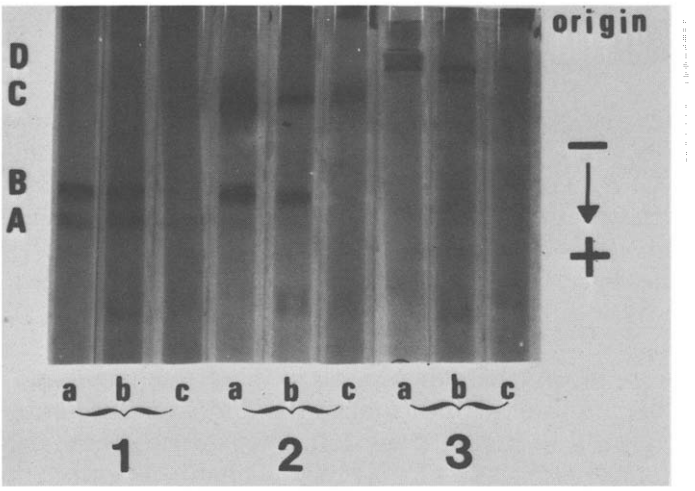

Fig. 8. Photograph of disc gel polyacrylamide electrophoresis [13]. 1, Human red cell NADH-methemoglobin reductase. 2, Soluble cytochrome b_5 reductase from human placenta. 3, Detergent-solubilized microsomal cytochrome b_5 reductase from human placenta. Prior to electrophoresis each enzyme preparation was preincubated with identical amounts of: (a) saline, (b) normal chicken serum, (c) chicken antiserum directed against human red cell methemoglobin reductase. The enzyme was stained as NADH-diaphorase [14].

methemoglobin reductase. After incubation with the different enzyme preparations, the precipitation lines were specifically stained for NADH-diaphorase [14]. As seen in Fig. 7, a continuous precipitation line without spur was found with the four compared enzyme preparations. This indicates that the four enzymes exhibit an identical immunoreactivity towards the anti-methemoglobin reductase. This result was further confirmed by electrophoresis following incubation of the enzymes with antiserum (Fig. 8). The bands A and B which are present in all preparations of soluble enzymes were completely suppressed (Fig. 8). Also band D found in preparation of microsomal enzyme, possessing very slow electrophoretic mobility, was completely abolished (Fig. 8). The additional band C existing in preparation of soluble cytochrome b_5 reductase from placenta was not completely inhibited, indicating the presence of at least another cross-reacting antigen with lesser affinity. This presence of band C can explain the additional slope found in the neutralisation test of the soluble placental enzyme.

Discussion

Originally NADH-cytochrome b_5 reductase has been found in microsomes from different tissues and in different species [21,22]. The microsomal NADH-cytochrome b_5 reductase is an amphipathic protein with a hydrophilic part and a hydrophobic part [23,24], the latter being responsible for the insertion of the enzyme in the endoplasmic membrane. Solubilization can be achieved by partial proteolysis by lysosomes or by trypsin yielding a molecule of about 28 000 daltons [25]. In contrast when the microsomal enzyme is solubilized by detergents such as Triton X-100 or deoxycholate, the intact molecule is obtained. The elementary subunit has a monomeric mol. wt. of about 33 000 [24], forming, in the absence of detergent, high mol. wt. aggregates, up to 300 000—600 000, and binding readily to membranous structures such as liver microsomes, liver mitochondria, erythrocyte ghosts and phosphatidylcholine liposomes [23,24,26].

In human red blood cells the NADH-cytochrome b_5 reductase is a soluble protein of less than 40 000 daltons which plays a role of methemoglobin-reductase [6]. A homozygous hereditary deficiency of the red-cell enzyme produces recessive congenital methemoglobinemia [1,2,3]. We also found a soluble "methemogobin-reductase-like" material in other human cells such as leukocytes, platelets, muscle, liver, brain and placenta [18]. In rat liver cytosol a NADH-cytochrome b_5 reductase-like enzyme was found by Zinsmeyer et al. [27].

In previous studies we have shown that when recessive congenital methemoglobinemia is associated with a severe neurologic disorder and mental retardation, the enzyme defect consists in a combined deficiency of cytosolic and microsomal NADH-cytochrome b_5 reductase [1,2,4]. It was therefore important to clarify the relationship between these two forms. Placenta was selected as a convenient source of human tissue to prepare both cytosolic and microsomal NADH-cytochrome b_5 reductase. In human red cells we prepared independently a methemoglobin reductase and a soluble NADH-cytochrome b_5 reductase. The four preparations were compared to each other, using essentially

two main criteria: electrophoretic mobility and immunological reactivity. Specific methods based upon the enzymatic function, NADH-diaphorase, NADH-methemoglobin reductase and NADH-cytochrome b_5 reductase, allowed us to carry out the characterizations in semi-purified preparations.

Our results clearly indicate that red-cell NADH-methemoglobin reductase and red-cell NADH-cytochrome b_5 reductase represent identical enzyme entities. They exhibit similar molecular weight (about 30 000), isoelectric pH and identical immunological reactivity towards anti-human red-cell methemoglobin reductase. Semi-purified soluble NADH-cytochrome b_5 reductase from human placenta also exhibits the same similarities with the red-cell enzyme. This indicates that, at least in red cells and in placenta, NADH-cytochrome b_5 reductase exists in a soluble form with a mol. wt. of approximately 30 000.

The detergent-solubilized microsomal NADH-cytochrome b_5 reductase prepared from human placenta was found to exhibit great similarities with the soluble NADH-cytochrome b_5 reductase. Qualitative tests such as double-immunodiffusion and electrophoresis after incubation with the anti-methemoglobin reductase antiserum are even compatible with a complete identity between the microsomal and the soluble enzymes. However they display a different electrophoretic pattern and a slightly different equivalence point. These differences may be explained by differences in the quaternary structure and by the tendency of the detergent solubilized enzyme to form aggregates.

It is tempting to correlate the soluble cytochrome b_5 reductase to the lysosomal digested microsomal cytochrome b_5 reductase or l-fp_1 of Takesue and Omura [25] for which a mol. wt. of 28 000 was found [25]. If we admit that both soluble and microsomal NADH-cytochrome b_5 reductase are coded by a single gene [4], the former could be a product of post-translational proteolytic degradation of the latter. The sequence: microsomal cytochrome b_5 reductase (monomeric mol. wt. 33 000) → soluble cytochrome b_5 reductase (mol. wt. 28 000) is likely, and would reproduce in vivo what is obtained in vitro by proteolytic digestion [25]. In most cells both forms would coexist, with the microsomal enzyme highly predominant. In red cells where subcellular structures have disappeared, only the soluble NADH-cytochrome b_5 reductase would remain and play a major role in methemoglobin reduction.

Note

After completion of this work, the papers of Kuma et al. [28] and Goto-Tamura et al. [29] appeared, showing also an immunological similarity between rat liver microsomal cytochrome b_5 reductase and animal and human red cell methemoglobin reductase.

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References

1 Leroux, A . and Kaplan, J.C. (1975) in VII. Internationales Symposium über Struktur und Funktion der Erythrozyten, Berlin 1973, pp. 349—354, Akad. Verlag, Berlin

2 Kaplan, J.C., Leroux, A., Bakouri, S., Grangaud, J.P. and Benabadji, M. (1974) Nouv. Rev. Fr. Hématol. 14, 755—770
3 Kitao, T., Sugita, Y., Yoneyama, Y. and Hattori, K. (1974) Blood 44, 879—884
4 Leroux, A., Junien, C., Kaplan, J.C. and Bamberger, J. (1975) Nature 258, 619—620
5 Hultquist, D.E. and Passon, P.G. (1971) Nat. New Biol. 229, 252—254
6 Passon, P.G. and Hultquist, D.E. (1972) Biochim. Biophys. Acta 275, 62—73
7 Omura, T. and Takesue, S. (1970) J. Biochem. 67, 249—257
8 Hegesh, E., Calmanovici, N.and Avron, M. (1968) J. Lab. Clin. Med. 72, 339—344
9 Hegesh, E. and Avron, M. (1967) Biochim. Biophys. Acta 146, 91—101
10 Scott, E.M. and McGraw, J.C. (1962) J. Biol. Chem. 237, 249—252
11 Zamudio, I. and Canessa, M. (1966) Biochim. Biophys. Acta 120, 165—169
12 Mihara, K. and Sato, R. (1972) J. Biochem. 71, 725—735
13 Ornstein, L. (1964) Ann. N.Y. Acad. Sci. 121, 151—163
14 Kaplan, J.C. and Beutler, E. (1967) Biochem. Biophys. Res. Commun. 29, 605—610
15 Drysdale, J.W., Righetti, P. and Bunn, H.F. (1971) Biochim. Biophys. Acta 229, 42—50
16 Zijlstra, W.G. and Von Kampen, E.J. (1961) Clin. Chim. Acta 6, 538—541
17 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
18 Leroux, A. and Kaplan, J.C. (1972) Biochem. Biophys. Res. Commun. 49, 945—950
19 West, C.A. (1969) Ph.D. Thesis, University of London
20 Ouchterlony, O. (1953) Acta Pathol. Microbiol. Scand. 32, 231—240
21 Strittmatter, P. (1963) In The Enzymes (Boyer, P., Lardy, H. and Myrbäck, K., eds.), 2nd edn., Vol. 8, p. 113, Academic Press, New York
22 Inouye, A. and Shinagawa, Y. (1965) J. Neurochem. 12, 803—813
23 Spatz, L. and Strittmatter, P. (1973) J. Biol. Chem. 248, 793—799
24 Mihara, K. and Sato, R. (1975) J. Biochem. 78, 1057—1073
25 Takesue, S. and Omura, T. (1970) J. Biochem. 67, 267—276
26 Rogers, M.J. and Strittmatter, P. (1974) J. Biol. Chem. 249, 5565—5569
27 Zinsmeyer, J., Schönfeld, W. and Wagenknecht, C. (1972) Acta Biol. Med. Germ. 28, 63—71
28 Kuma, F., Prough, R.A. and Masters, B.S.S. (1976) Arch. Biochem. Biophys. 172, 600—607
29 Goto-Tamura, R., Takesue, Y. and Takesue, S. (1976) Biochim. Biophys. Acta 423, 293—302