Biochimica et Biophysica Acta, 613 (1980) 34—40

BBA 68953

PURIFICATION AND CHARACTERIZATION OF A NOVEL HUMAN RED CELL ENZYME WITH DIPHENOL OXIDASE ACTIVITY

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(Received July 31st, 1979)

Key words: Diphenol oxidase; Adrenalin; Dihydroxyphenylalanine; (Human red cell)

Summary

A new enzyme protein, diphenol oxidase, has been isolated and purified from human red cells and catalyzes the in vitro formation of adrenochrome from epinephrine and of melanin from dihydroxyphenylalanine. Some immunological and physicochemical properties of this protein have been studied.

Introduction

A factor which catalyzes the in vitro formation of adrenochrome from epinephrine and of melanin from dihydroxyphenylalanine has been described in human platelets [1]. This factor, called diphenol oxidase (1,2-benzenediol:oxygen oxidoreductase, EC 1.10.3.1) shows some similarities with DOPA oxidase [2] and epinephrine-oxidizing enzyme [3,4]. Some abnormalities of this diphenol oxidase have been observed in human muscular dystrophy [5].

The diphenol oxidase activity has now been found in most animal tissues [6], particularly in the kidney, the blood platelets and the red cells (both in the cytosol and membranes [7,8]).

We have now purified and characterized the human red cell enzyme.

Materials and Methods

1. Material

Human red cells freed from platelets and leukocytes were kindly provided by the Centre National de Transfusion Sanguine.

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Abbreviation: DOPA, 3,4-dihydroxyphenylalanine.

All reagents were from the highly purified grade: Tris-HCl from Merck; maleic acid (puris) and β-L-3,4-dihydroxyphenylalanine (puris) from Sochibo; L-epinephrine bitartrate from Sigma; DEAE-Sephadex from Pharmacia; Ampholines and Ultrodex from LKB.

2. Assay of the diphenol oxidase activity

The substrate was $2.2 \cdot 10^{-3}$ M L-epinephrine bitartrate (final molarity) in 0.375 M Tris-HCl (pH 9.55) buffer and the adrenochrome formed at 37°C in this buffer was assayed by the method of Démos [5]. The activity was measured exactly 15 s after addition of the extract to the substrate. Measurements were recorded with an automatic spectrophotometer (PYE Unicam SP 8-200) connected to a computer (Hewlett Packard 9825 A). The adrenochrome formed was assayed by its absorption at 485 nm. The values of the blanks were substracted, as in the case of the cytochrome oxidase (which also has an action on an autoxidizable substrate [9,10]). The blank values are high because (i) the substrate is autoxidizable and (ii) hemoglobin, when present, possesses an intrinsic oxidizing action. Therefore, whenever hemoglobin is present in the sample to be assayed, a separate blank was prepared with hemoglobin freed from diphenol oxidase (first step of purification, see below).

The enzyme activity is expressed in international units (I.U.) at 485 nm per min per mg protein.

3. Determination of hemoglobin

This was done by the method of Zijlstra and van Kampen [11].

4. Determination of proteins

Proteins were determined in diluted solutions (less than 1 mg/ml) by measuring the 280 nm/260 nm absorbance ratio. Absorption was always maximal at 280 nm and the formula of Warburg and Christian was used [12].

5. Electrophoretic studies

Regular electrophoresis was performed in 12 × 0.5 cm tubes according to Ornstein [13] and sodium dodecyl sulfate (SDS) electrophoresis according to Weber and Osborn [14]. Slab gel electrophoresis was made with a linear concentration gradient of 2.5—28% polyacrylamide [8]. Isoelectrofocusing was performed in slab polyacrylamide gels using plates manufactured by LKB (gradient pH 3.5—9.5 or 5—8) or in tubes (12 × 0.5 cm) according to Drysdale et al. [15].

6. Immunological studies

(a). Preparation of the antiserum. Seven intradermic injections of 200 μg purified fraction, were performed every 10 days on rabbits (2.8 kg mean weight).

The antigen was distributed over 20 injection sites.

(b). Ouchterlony double immunodiffusion [16]. The antiserum was inactivated by heating at 56°C for 2 h and deposited in the center well of a plating dish. The antigens at different protein concentrations were added to surrounding wells. The precipitin lines were formed after 48 h at 4°C. The agar

was washed with an isotonic NaCl solution and stained with Coomassie blue or specifically with dihydroxyphenylalanine.

7. Staining methods used after electrophoresis or Ouchterlony immunodiffusion

(a). Specific staining of diphenol oxidase. 0.01 M dihydroxyphenylalanine was used according to the method of Démos [1,5]. The activity was detected by the appearance of black spots corresponding to local formation of melanin.

(b). Protein staining. Proteins were stained with Coomassie blue according to the method of Neville [17].

8. Purification of the diphenol oxidase

(a). Preparation of hemolysate. The red cells were washed three times with 0.9% NaCl and lysed with 2 vols. distilled H_2O.

(b). Preparation of a non-heme protein extract. The hemolysates were freed from hemoglobin by batch adsorption on DEAE-Sephadex A50, a method modified from our earlier work [18]: the hemolysate was buffered with 0.05 M Tris-HCl (pH 8.46)/0.02% sodium nitrite (v/v) and then dialyzed with the same buffer. DEAE-Sephadex A50 was added (20 g dry weight resin for 10 g hemoglobin). Hemoglobin was eluted from the resin with 0.05 M Tris-HCl (pH 7.4) buffer. The other protein components (including diphenol oxidase) were eluted afterwards by washing with 0.05 M Tris-maleate (pH 4.23), three times.

These eluates were concentrated (Diaflo membrane PM 10), dialyzed against distilled water and centrifuged at $30\,000 \times g$ for 15 min. All steps were performed at 4°C.

(c). Flat bed preparative isoelectrofocusing. The procedure described by LKB was used (length 24.5 cm, width 11 cm, thickness 0.52 cm). A mixture of Ultrodex (LKB) and ampholines (pH 5—8 range) was prepared and evaporated until 28% H_2O was lost. 7 mg proteins were mixed with the gel containing the ampholines and inserted 10 cm from the cathode. The electrophoresis was done at constant power 8 W, initial voltage 600 V. After a 16 h run, the gel was sliced into 30 fractions, corresponding approx. to intervals of 0.1 pH units.

The fractions were eluted with distilled water and the linearity of the pH gradient was measured. The ampholines were removed by extensive dialysis against distilled water or Tris-magnesium citrate (pH 8.7). Final concentration was performed by ultrafiltration with a Diaflo PM 10 membrane.

Results

Validity of the assay

The diphenol oxidase was detected in fractions 16—22 (pH 6.3—5.8), because of the diffusion of proteins in Ultrodex. However a weak diphenol oxidase activity was observed in fractions 23—30 (pH 5.7—5) containing ten other contaminants (major contaminant was superoxide dismutase which has acidic pH_i).

The diphenol oxidase activity of the final preparation (fractions 16—22) increased 1200 times, with a 32% yield compared to the red cell lysate

TABLE I

MEASUREMENTS OF ACTIVITY RATE OF THE HUMAN RED CELL DIPHENOL OXIDASE AFTER EACH STEP OF THE PURIFICATION PROCEDURE (34 g RED CELLS)

	Volume (ml)	Total protein (mg)	Specific activity (I.U.)	Total activity (I.U.)	Yield in total activity (%)
Crude extract red cell lysate in sodium nitrite	204	984 *	56	55 677	100
Fraction after DEAE-Sephadex A50 adsorption	1.5	6.93	6 893	47 752	85
Final fraction after purification by preparative isoelectrofocusing (pH 5—8)	0.7	0.252	70 163	17 682	31.7

* Hemolysate protein - hemoglobin eluted from the batch.

(Table I). However, a precise estimation of the recovery and the extent of purification cannot be achieved, since the initial measurements of the activity in the presence of hemoglobin are not accurate. The oxidizing activity was proportional to the quantity of the purified protein added (between 20 and 40 μg protein, results not shown) to the assay mixture.

The factor is completely inactivated by heating at 100°C for 10 min or by proteolysis with pronase at 5% in 0.05 M Tris-HCl (pH 7.4) buffer (results not shown).

These properties, in addition to the fact that the oxidizing factor is not dialyzable show that this factor is an enzyme protein.

Purification

The following criteria suggest that the final enzyme preparation was pure:

1. Electrophoresis on polyacrylamide gel showed only one diphenol oxidase activity band as revealed by dihydroxyphenylalanine, which coincided with a single protein band (as stained by Coomassie blue) of about M_r 150 000 (hemoglobin, aldolase and catalase were used as standard markers).

2. Electrophoresis on SDS-polyacrylamide gels showed a single band of about M_r 75 000. (Bovine serum albumin and catalase were used as standard markers).

3. Double immunodiffusion according to Ouchterlony [16] gave a single precipitin line which does not have any action upon dihydroxyphenylalanine (Fig. 1).

Partial characterization

Molecular weight estimation: electrophoresis on 2.5—28% polyacrylamide gradient gel showed, after incubation with dihydroxyphenylalanine, two active bands close to each other but defined. These are observed at all stages of the purification procedure: total lysate, after DEAE-Sephadex purification and the final sample (Fig. 2). Isoelectric pH: isoelectrofocusing in polyacrylamide gel has given the pH_i values of these two bands, from the purified sample as well

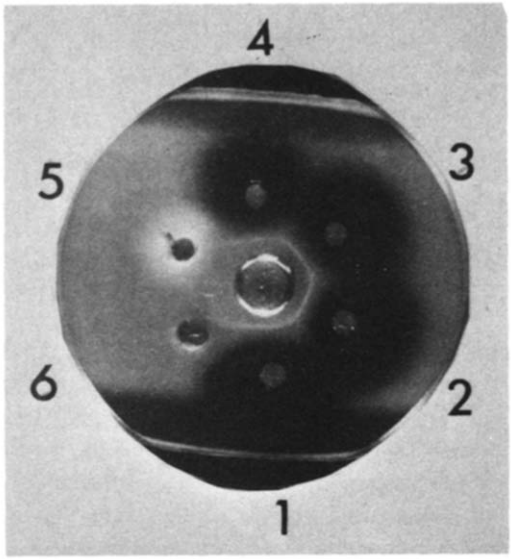

Fig. 1. Double immunodiffusion according to Ouchterlony with rabbit antiserum in the center of the plating dish, purified diphenol oxidase at different concentrations and rabbit serum all around. 1, 2, 3, 4: final purified fraction at different concentrations. 1, 0.5 mg/ml; 2, 0.26 mg/ml; 3, 0.13 mg/ml; 4, 0.05 mg/ml; 5, 6, rabbit serum: 5, non-diluted; 6, 1/10 time diluted. The precipitin line was formed after 48 h at 4°C. The plating dish was washed with a NaCl isotonic solution and incubated in dihydroxyphenylalanine.

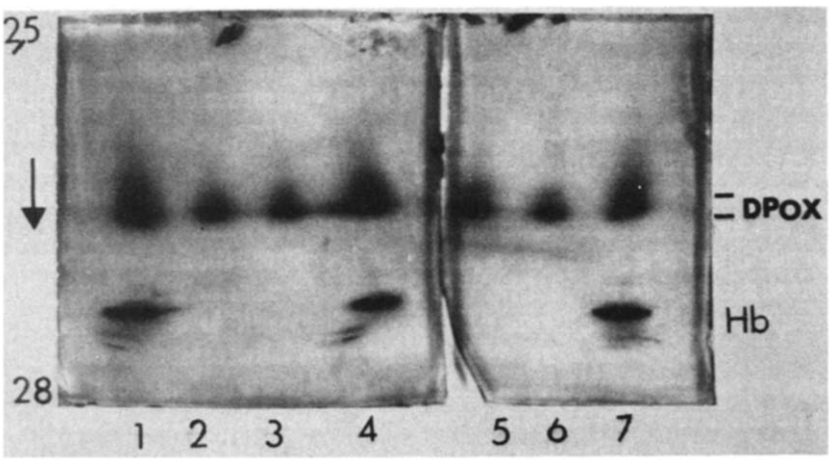

Fig. 2. Slab electrophoresis with a linear gradient of polyacrylamide (2.5—28%). Pattern observed throughout the different purification steps. 1, 4, 7, hemolysate of red cells (46 mg/ml of proteins); 2, 3, protein fraction after the batch of DEAE-Sephadex A50 (0.25 mg/ml of proteins); 5, 6, purified protein fraction (0.01 mg/ml of proteins). After electrophoresis the plate is incubated in dihydroxyphenylalanine. DPOX: diphenol oxidase.

as from the crude extract at pH 6.6 and 6.8 for the major and minor bands, respectively.

Substrates: the purified fraction has a catalytic action on L-dihydroxyphenylalanine and L-epinephrine, but not on tetrazolium blue (which is a superoxide dismutase substrate). The purified fraction has no effect on tyrosine, a substrate of tyrosinase.

Immunological properties: the purified human red cell antidiphenoloxidase antiserum from rabbit gave on a precipitin line with the purified fraction (Fig. 1). This antiserum inhibited the diphenol oxidase activity of the purified fraction.

Discussion

The enzyme and protein nature of the diphenol oxidase has been shown by thermal and proteolytic inactivation as well as by immunological precipitation and inactivation.

The method of purification yielded a single protein, as judged from polyacrylamide gel electrophoresis without and with SDS (M_r 150 000 for the native enzyme). This is in agreement with the value found previously in crude preparations of hemolysate and red cell membranes [8]. It is not yet possible to elucidate whether the two 75 000 subunits are identical.

Another problem which remains to be solved is the molecular significance of the electrophoretic heterogeneity of the enzyme, which was detected throughout the purification procedure.

After isoelectrofocusing and electrophoresis in an acrylamide gradient, there was more than one entity (one major, one minor) copurifying together; they may represent another example of microheterogeneity of a single protein [19].

The question of the physiological significance of this enzyme is of some interest: this enzyme has diphenol oxidase properties, since it increases the rate of in vitro oxidation of catecholamines such as epinephrine and dihydroxyphenylalanine. No similar enzyme has been reported before. The only known enzymes which are similar in some properties to the diphenol oxidase, are the epinephrine-oxidizing enzyme of Axelrod [3] and Inchiosa and Rodriguez [4], and the DOPA oxidase of Roskin and Grunbaum [2]. It is possible that, in the red cells, diphenol oxidase plays a role in the in vivo oxidation of catecholamines [20].

Acknowledgements

We wish to thank Professor J.C. Kaplan for his constant interest and valuable advice. We are very grateful to Mrs. Elghouzzi, Miss Delcey, Mr. Cavalier and all the staff of the deep-freeze laboratory of the 'Centre National de Transfusion Sanguine' in Paris, for the red cell preparations. We are thankful to the 'Institut National de la Santé et de la Recherche Médicale' (ATP 54-77-86) which supported this work. We are indebted to the 'Comité Français de Soutien à la Recherche contre la Myopathie' and the 'Union des Myopathes de France' for the funds they provided for us. The technical collaboration of Miss M. Berthelon, Miss N. Prémont and Miss Dautréaux was greatly appreciated.

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