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DIAPHORASE P: A NEW FETAL ISOZYME IDENTIFIED IN HUMAN PLACENTA

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Summary

Human placenta contains a thermostable, cytosolic NADH-diaphorase which is different from the other diaphorases and which we designate as diaphorase P.

It is specific for NADH and reduces artificial substrates such as dichlorophenol and tetrazolium derivatives, but not natural substrates such as methemoglobin, cytochrome b_5 or lipoate.

It is antigenically distinct from the ubiquitous red-cell type NADH-diaphorase (soluble cytochrome b_5 reductase) specified by the DIA_1 locus.

Using electrophoretic and immunologic methods, it was possible to detect diaphorase P in various fetal tissues (brain, liver, kidney, muscle), whereas it was not found in adult tissues with the exception of the brain. This enzyme, the physiological role of which remains unknown, appears to belong, therefore, to the category of fetal proteins. Its resurgence in primary liver cancer was demonstrated in three cases.

Introduction

Human red cells contain a NADH-diaphorase which plays a major role in the reduction of methemoglobin [1]. This enzyme is also widely distributed in many non-erythroid tissues [2] and corresponds to NADH-cytochrome b_5 reductase [3], specified by a locus designated diaphorase 1 (or DIA_1, Fisher et al. [4]). During a study of the tissue distribution of this enzyme, using starch gel electrophoresis followed by specific staining [5], we found that human

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Abbreviation: MTT, 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyltetrazolium bromide.

placenta contained, in addition to the ubiquitous NADH-diaphorase 1, a slower moving and thermostable NADH-diaphorase (diaphorase P) [6]. In this study we have characterized and partially purified this enzyme. Evidence is given that diaphorase P is different from the other diaphorases, and characteristic of fetal, and possibly cancerous, tissues.

Materials and Methods

Fresh, normal, human placentas, obtained immediately after delivery, were minced and thoroughly washed with isotonic, cold sucrose. After homogenization in a Waring Blendor, the preparation was freed from cellular debris by several centrifugation steps. The 30 000 $\times g$ supernatant was used as starting material for all subsequent studies.

The other tissues studied were homogenized in a Potter-Elvejhem apparatus using 3 vols. 0.066 M sodium phosphate/potassium phosphate (1 vol. Na_2HPO_4: 10 vols. KH_2PO_4) buffer (pH 5.9) containing 1 mM EDTA and subsequently centrifuged at 30 000 $\times g$.

Horizontal starch gel electrophoresis was performed as described by Kaplan and Beutler [5] using a Tris/EDTA/borate buffer (pH 8.6) as recommended by Hopkinson et al. [7]. Polyacrylamide gel electrophoresis was performed according to the method of Ornstein [8]. Polyacrylamide gel isoelectrofocusing was carried out in a pH 3—8 linear gradient (Ampholines, LKB) according to the method of Drysdal et al. [9]. In all the electrophoretic studies, specific staining for NADH-diaphorase activity was performed with a mixture containing 1.2 mM NADH, 0.06 mM dichlorophenol indophenol and 1.2 mM 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) in a 0.25 M Tris-HCl buffer (pH 8.4) as described previously [5]. However it was found that dichlorophenol indophenol could be omitted without affecting the intensity of the diaphorase P, whereas other diaphorase bands (such as diaphorase 1) were considerably decreased.

Enzyme assays

The NADH-diaphorase activity was assayed with dichlorophenol indophenol as an acceptor, according to Scott and McGraw [10]. The NADH-methemoglobin reductase activity was assayed using the method of Hegesh et al. [11], in which the acceptor is a ferrocyanide-methemoglobin complex.

A special assay (tetrazolium-reducing assay) was devised for the diaphorase P, when it was found that the enzyme unexpectedly promoted the reduction of the tetrazolium derivative (MTT), in the absence of an intermediate electron carrier. The assay mixture contained 1.5 mM NADH and 2.4 mM MTT in a 0.25 M Tris-HCl buffer (pH 8.4). The reaction was started with NADH and followed by measuring the absorbance change at 578 nm. The molecular extinction coefficient of reduced MTT was found to be 8.0. One unit is defined as 1 μmol of MTT reduced per min, on the basis of one electron transferred. Although not strictly specific for diaphorase P, this procedure was found to be more accurate and sensitive than conventional methods.

Sedimentation coefficient

Sedimentation coefficient was estimated by sucrose gradient density centri-

fugation according to Martin and Ames [12]. The marker proteins were glucose oxidase (7 S), hemoglobin (4.5 S) and cytochrome *c* (1.9 S).

Preparation of antidiaphorase P antiserum

The best preparation of semi-purified diaphorase P was injected into one rabbit. The total amount of antigen corresponded to 1 mg of protein (1000 munits), and was divided into 20—30 intradermal injections. This procedure was repeated four times at intervals of 3 weeks. The antiserum was collected and kept frozen. Double immunodiffusion was performed according to Ouchterlony [13]. Antigens and antiserum were incubated for 2—3 days, the plate was then extensively washed with isotonic saline for 2 days to remove the excess enzyme that had not reacted with the antiserum. The precipitation lines were specifically visualized using the NADH-diaphorase staining method [5], with or without dichlorophenol indophenol.

Purification of diaphorase P

The 30 000 × *g* supernatant of placenta homogenate was sequentially precipitated by ammonium sulfate at saturations of 0—30%, 30—35% and 50—70%. Diaphorase P was detected by its electrophoretic mobility in the 50—70% saturation precipitate. This fraction was redissolved in the buffer used for preparing the homogenate. The now more concentrated enzyme salted out at a 40% saturation of ammonium sulfate. The precipitate was dialyzed for 24 h against the same buffer, centrifuged at 30 000 × *g*, and the supernatant chromatographed on a CM-Sephadex 50 column equilibrated with the same buffer. Under these conditions the major NADH-diaphorase activity corresponding to the red-cell type enzyme (soluble cytochrome b_5 reductase) was not retained by the column and was eliminated during the washing step. Diaphorase P was eluted with a linear gradient (0—0.35 M) of KCl dissolved in the same buffer. It was detected as a single peak at 0.15 M KCl. The active fractions were pooled and concentrated by vacuum dialysis against 50 mM Tris-HCl buffer, containing 1 mM EDTA (pH 8.0). The dialyzed fractions was then chromatographed on a DEAE-Sephadex A50 column equilibrated with the dialyzing buffer. Diaphorase P was eluted with a linear gradient of 0—0.80 M KCl in the same buffer. The enzyme appeared as a single peak at approx. 0.4 M KCl and was concentrated by vacuum dialysis. It could be stored at —20°C for at least 2 months without loss of activity.

Because of the lack of specificity of the enzyme assay, the presence of diaphorase P was monitored at all purification steps by combining electrophoresis and diaphorase activity measurement.

For the same reason it was not possible to calculate the actual purification of diaphorase P, since the quantitative measurements carried out during the procedure were biased by the interference of the other diaphorases.

Results

The specific activity of the best final preparations was 1500—2000 munits per mg protein. The purity was checked by polyacrylamide gel electrophoresis. Protein staining showed a major band with the same mobility as the band

obtained by specific staining for the enzyme activity. Very faint bands of contaminating proteins were seen, however, indicating that the preparation was not quite homogeneous.

Properties of the semi-purified diaphorase P

Starch gel electrophoresis followed by specific staining. Since the enzyme was first discovered as the slowest moving NADH-diaphorase during starch gel electrophoresis of human placental crude extracts [6], the criterion of electrophoretic mobility was used for monitoring the enzyme purification throughout all steps. During starch gel electrophoresis, it migrated as a single band cathodal to the ubiquitous red-cell type NADH-diaphorase (DIA_1) (Fig. 1).

Specificity for reduced coenzyme. Using the tetrazolium reducing assay, with or without dichlorophenol indophenol as intermediate, the enzyme was strictly specific for NADH. It exhibited no activity with NADPH, neither in the assay nor upon electrophoresis. The apparent Michaelis constant for NADH in the presence of 2.4 mM MTT at pH 8.4 was 68 μM.

Specificity for electron acceptors. Although dichlorophenol indophenol was reduced by the enzyme in the presence of NADH [10], it did not give reproducible assays. In contrast, our assay with tetrazolium reduction gave satisfactory results in terms of linearity according to time and enzyme concentration. The apparent K_m for MTT was 134 μM. The diaphorase P displayed a very weak methemoglobin reductase activity [11] and no reducing activity towards potassium ferricyanide [14], lipoate [15] and cytochrome b_5 [16] (Table I).

Isoelectric pH. The p*I*, as measured by polyacrylamide gel isoelectrofocusing [9], was 6.95 ± 0.05.

Influence of pH. The enzyme exhibited a maximal NADH-tetrazolium reducing activity between pH 8 and 9.

Thermostability. Diaphorase P was found to be very thermostable. After being heated at 60° C in the presence of 1 g/l bovine serum-albumin, the semi-

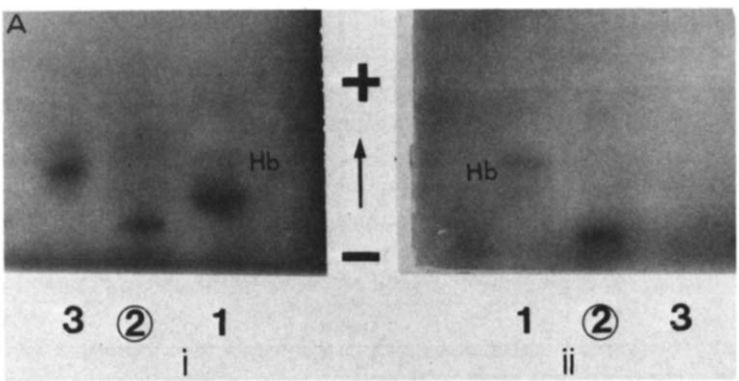

Fig. 1. Starch gel electrophoresis of placenta diaphorase P and red cell DIA 1. Electrophoresis in 12% starch gel in Tris/EDTA/borate buffer (pH 8.6). Migration for 18 h at 200 vols. Staining with NADH according to Kaplan and Beutler [5]. 1, fresh hemolysate (only DIA 1 is visible); 2, semi-purified diaphorase P; 3, purified red-cell DIA 1; i, unheated gel; ii, gel heated at 60° C for 15 min after electrophoresis and before staining [17].

purified preparation still retained 75% of initial activity after 60 min, and 55% after 90 min. In contrast, when heated in similar conditions, a purified preparation of red-cell cytochrome b_5 reductase was completely inactivated within 15 min. Similar results were obtained when heat was applied to the gel after electrophoresis of unheated extracts [17] (Fig. 1).

Effect of some inhibitors

Diaphorase P was completely inhibited by incubation with $5 \cdot 10^{-4}$ M *p*-chloromercuribenzoate for 15 min at room temperature, indicating its requirement for intact SH group. The enzyme was not affected by 0.01 mM dicoumarol, 1 mM amytal or 1 mM KCN.

Estimation of molecular weight

After ultracentrifugation in a linear concentration gradient of sucrose (5—20%), diaphorase P displayed a sedimentation coefficient of 6.6 S as compared to glucose-oxidase (7 S), hemoglobin (4.5 S) and cytochrome *c* (1.9 S) [12]. This value corresponds to an approximate molecular weight of 130 000.

Subcellular distribution

After separating the crude homogenate into several subcellular fractions, the diaphorase P, as detected by electrophoresis, was found only in the 150 000 $\times g$ supernatant. This enzyme is therefore strictly cytosolic.

Comparison with soluble red-cell cytochrome b_5 reductase (NADH-diaphorase or diaphorase 1)

Immuno-inactivation, and double immunodiffusion according to Ouchterlony [13], using an antiserum directed against the soluble red cell cytochrome b_5 reductase, showed that diaphorase P was insensitive to this antiserum (data not shown).

Tissue distribution

In order to determine whether the diaphorase P was specifically placental, a search was made for the enzyme in several tissues of either fetal or adult origin. Crude extracts were investigated using two methods: (i) electrophoresis followed by specific staining, (ii) immunoprecipitation according to Ouchter-

TABLE I

ACTIVITY OF DIAPHORASE P UPON SOME ELECTRON ACCEPTORS

Acceptor	Velocity (nmol/min per mg protein)	
	With NADH	With NADPH
MTT	2850	0
Dichlorophenol inodophenol	475	0
Methemoglobin-ferrocyanide complex	2.5	0
Ferricyanide	0	0
Lipoate	0	0
t-Cytochrome b_5	0	0

lony [13], followed by specific staining, a method more specific and more sensitive than the former. Using both criteria, diaphorase P was detected in all fetal tissues explored: brain, muscle, liver, lung, kidney. A reaction of identity with placental purified diaphorase P was obtained on the Ouchterlony plates. In contrast, diaphorase P was not found by the two methods in adult tissues (liver, muscle, kidney, lung, erythrocyte and leukocytes). However, in adult brain, traces of diaphorase P could be detected only by the immunological method.

Although it was detected in placenta at an early stage (first trimester), the enzyme was not found in the serum during pregnancy, nor was it found in fibroblasts deriving from cultured amniotic cells obtained at the 12th week of pregnancy.

Detection in human primary hepatomas

Three surgical samples of human primary hepatoma were investigated using Ouchterlony's double immunodiffusion technique followed by specific staining for NADH-diaphorase activity. A single precipitate line retaining diaphorase activity was seen for human hepatoma, in continuity with the diaphorase P line from both crude extract and purified placenta preparations. In contrast, there were no lines with normal adult liver extract (Fig. 2).

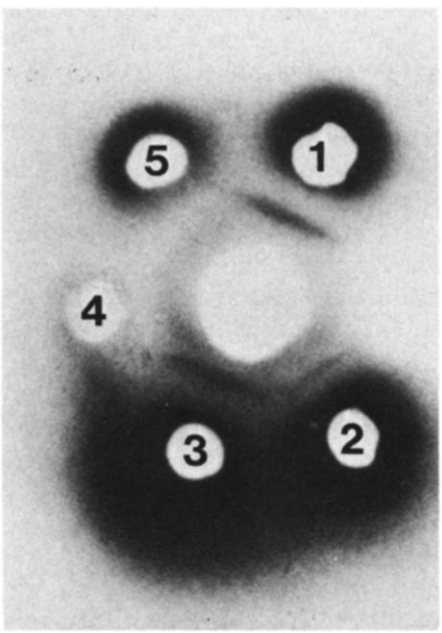

Fig. 2. Double immunodiffusion according to Ouchterlony [13], in 1% agarose containing 0.15 M NaCl. The central well contains the anti-diaphorase P antiserum. After 2—3 days incubation and 2 days extensive washing with isotonic saline the plate was stained for NADH-diaphorase activity [5]. Each line therefore represents an immunoprecipitate retaining diaphorase activity. Note the absence of cross reactivity with the red-cell main diaphorase (DIA_1 or NADH cytochrome b_5 reductase). (1) semi-purified diaphorase P; (2) crude extract of placenta; (3) human primary hepatoma; (4) purified red-cell NADH cytochrome b_5 reductase; (5) human normal adult liver.

Discussion

Among the diaphorases already described, diaphorase P appears as a new and unique entity. Because of its cytosolic localization and NADH requirement, it can only be compared to the known soluble NADH-diaphorases. The only well-characterized, soluble NADH-diaphorase was found initially in red cells [10], where it promotes methemoglobin reduction. It was later detected in almost all cells and tissues [2,3] including the placenta, and is now considered to be a soluble form of NADH-cytochrome b_5 reductase [2,18]. This entity is easily distinguishable from diaphorase P by a variety of parameters: molecular weight, isoelectric pH, thermostability. Furthermore, there is no antigenic relationship between the two enzymes: (i) diaphorase P does not crossreact with an antiserum directed against the red-cell NADH-diaphorase (cytochrome b_5 reductase), (ii) the red-cell NADH-diaphorase does not react with an antiserum directed against diaphorase P (Fig. 2). During the course of purification of diaphorase P from placental extracts in which the red-cell type NADH-diaphorase coexists, both entities could be readily separated. They thus appear to be different enzymes.

The other diaphorases exhibit different properties. The fact that diaphorase P does not accept NADPH rules out any similarity with: (i) NADPH-diaphorase (DIA_2, Fisher et al. [4]) which also has a very different electrophoretic mobility; (ii) NADPH cytochrome *c* reductase; (iii) DT-diaphorase [19] which is inhibited by dicoumarol; (iv) with the diaphorase 3 isozyme (from the DIA_3 locus [4,20]).

The physiological significance of diaphorase P remains obscure, as long as a natural electron acceptor is not found for the enzyme. It only acts upon xenobiotic acceptors, and the enzyme could be tentatively categorized as a NADH-tetrazolium reductase. A nitro-blue tetrazolium reductase was described in human polymorphonuclear leukocytes [21]. Its cellular distribution, essentially microsomal, and coenzyme specificity (it accepts equally NADH and NADPH) are different from those of diaphorase P. Furthermore diaphorase P was not found in human leukocytes, a rich source of the nitro-blue tetrazolium reductase [21]. Any relationship between the two enzymes can therefore be ruled out.

Accordingly, diaphorase P cannot be the diaphorase isozyme first discovered by Caldwell et al. [20] in sperm, and further investigated (and designated DIA_3) by Fisher et al. [4], because the latter is thermolabile, accepts NADPH, and has a more alkaline isoelectric pH (p*I* 7.9).

The main interest of diaphorase P resides in its ontogenic significance. Primarily found in placenta, it was readily detected in fetal tissues and, therefore, appears as a fetal isozyme. Its absence in all adult tissues, except the brain, is reminiscent of the distribution of some other fetal isozymes [22].

Moreover, we found diaphorase P in three cases of adult human primary liver cancer, whereas it was not found in control adult human liver. Since the pioneer work of Schapira et al. [23] on aldolase in primary liver cancer, many fetal enzymes have been found in cancerous tissues and classified as 'carcino-fetal' proteins [22]. Among them is the much investigated, thermostable alkaline phosphatase from placenta, or 'Regan isozyme' [24]. Diaphorase P might belong to the same category.

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References

1 Schwartz, J.M. and Jaffé, E.R. (1978) in The Metabolic Basis of Inherited Disease (Stanbury, J.S., Wyngaarden, J.B. and Frederickson, O.S., eds.), 4th edn., pp. 1452—1464, McGraw-Hill, New York
2 Leroux, A. and Kaplan, J.C. (1972) Biochem. Biophys. Res. Commun. 49, 945—950
3 Leroux, A., Torlinski, L. and Kaplan, J.C. (1977) Biochim. Biophys. Acta 481, 50—62
4 Fisher, R.A., Edwards, Y.H., Putt, W., Potter, J. and Hopkinson, D.A. (1977) Ann. Hum. Genet. 41, 139—149
5 Kaplan, J.C., and Beutler, E. (1967) Biochem. Biophys. Res. Commun, 29, 605—610
6 Kaplan, J.C., Choury, D. and Leroux, A. (1974) C.R. Acad. Sci. 278, 393—395
7 Hopkinson, D.A., Corney, G., Cook, P.J.L., Robson, E.B. and Harris, H. (1970) Ann. Hum. Genet. 34, 1—10
8 Ornstein, L. (1964) Ann. N.Y. Acad. Sci. 121, 151—163
9 Drysdale, J.W., Righetti, P. and Bunn, H.F. (1971) Biochim. Biophys. Acta 229, 42—50
10 Scott, E.M. and McGraw, K.C. (1962) J. Biol. Chem. 237, 249—252
11 Hegesh, E., Calmanovici, N. and Avron, M. (1968) J. Lab. Clin. Med. 72, 339—344
12 Martin, R.G. and Ames, B.N. (1961) J. Biol. Chem. 236, 1372—1379
13 Ouchterlony, O. (1953) Acta Pathol. Microbiol. Scand. 32, 231—240
14 Zamudio, I. and Canessa, M. (1966) Biochim. Biophys. Acta 120, 165—169
15 Massey, V. (1963) in The Enzymes (Boyer, P.D., Lardy, H. and Myrbäck, K., eds.), Vol. 7, pp. 275—306, Academic Press, New York
16 Mihara, K., and Sato, R. (1972) J. Biochem. (Tokyo) 71, 725—735
17 McAlpine, J.P., Hopkinson, D.A., and Harris, H. (1970) Ann. Hum. Genet. 34, 61—71
18 Passon, P.G. and Hultquist, D.E. (1972) Biochim. Biophys. Acta 275, 62—73
19 Ernster, L., Ljunggren, M. and Danielson, L. (1960) Biochem. Biophys. Res. Commun. 2, 88—92
20 Caldwell, K., Blake, E.T. and Sensabaugh, G.F. (1976) Science 191, 1185—1187
21 Baehner, R.L. (1975) J. Lab. Clin. Med. 86, 785—792
22 Schapira, F. (1973) Adv. Cancer Res. 18, 77—153
23 Schapira, F., Dreyfus, J.C. and Schapira, G. (1963) Nature 200, 995—997
24 Fishman, W.H., Inglis, N.R., and Green, S. (1971) Cancer Res. 31, 1054—1057