BBA 63316

Intramitochondrial distribution of multiple forms of pig heart lipoamide dehydrogenase

Lipoamide dehydrogenase (NADH:lipoamide oxidoreductase, EC 1.6.4 3) is associated structurally and functionally with the mitochondrial α-keto acid dehydrogenase multienzyme complexes[1,2]. In addition, a free mitochondrial lipoamide dehydrogenase has been reported[3] Multiple forms have been isolated from pig heart[4,5] and beef heart and liver[3,6] using zonal electrophoresis and ion exchange chromatography

The present communication describes the electrophoretic properties on polyacrylamide gel discs of lipoamide dehydrogenase obtained by urea resolution of purified pig heart pyruvate and α-ketoglutarate dehydrogenases and a further study of the electrophoretic characteristics of particulate pig heart lipoamide dehydrogenase (Straub diaphorase) Multiple forms typical of the enzyme derived from each of the complexes are distinguishable and may be correlated with components characteristic of the Straub diaphorase

Pyruvate and α-ketoglutarate dehydrogenases were isolated from pig heart mitochondrial extracts essentially as described by HAYAKAWA *et al.*[7] and HIRASHIMA *et al.*[8] The corresponding enzymes from *Escherichia coli* K12 were isolated by procedures described by REED AND WILLMS[9] Lipoamide dehydrogenase was resolved from the purified α-keto acid dehydrogenases by incubating at 4° in 6 M urea, freshly prepared from recrystallized urea, at pH 7 5 for at least 2 5 h followed by chromatography on a calcium phosphate gel–cellulose column[10] to obtain the enzyme Straub diaphorase was prepared and assayed as described by MASSEY[10,11], using DL-lipoamide in the enzyme assay. Assay results are expressed as μmoles NADH oxidized by lipoamide per min Protein was determined[12] using bovine serum albumin as the standard and by following absorbance at 280 mμ.

Continuous polyacrylamide gel disc electrophoresis was performed essentially as described by CLARKE[13] using 4% acrylamide and *N*,*N*-methylenebisacrylamide in an amount to obtain 1% crosslinkage Electrophoresis was carried out in a unit (Buchler) containing 0.005 M Tris–HCl buffer (pH 8.5), cooled to 3° at a current of 1.5 mA/tube for 1 5–3 h Sample movement was to the anode. The electrophoresed gel discs were placed either in a solution containing 0 3 mg nitro-blue tetrazolium chloride and 0.6 mg NADH per ml 0 1 M phosphate buffer (pH 7 5) for 30 min to detect diaphorase activity, or in a solution of 10% trichloroacetic acid followed by transfer to 0 2% Coomassie blue[14] to visualize protein. In order to recover enzyme protein, 3-mm gel sections were placed on a 2-cm column of polyacrylamide gel which was polymerized over 0 4 ml 20% sucrose A dialysis membrane was fastened over the distal end of the tube. The sections were then electrophoresed and protein was collected in the sucrose solution, followed by assay for lipoamide dehydrogenase

Lipoamide dehydrogenase (Straub diaphorase) shows a single symmetrical peak when centrifuged at 59 780 rev /min in a Model E Spinco analytical ultracentrifuge. The sedimentation coefficient, $s_{20,w}$, is 5 4 (4 1 mg protein per ml) and the absorbance ratio, $A_{280\ m\mu}/A_{455\ m\mu}$, is 5 33, in agreement with data reported by MASSEY[10] Continuous gel disc electrophoresis of this preparation gives the diaphorase-reactive

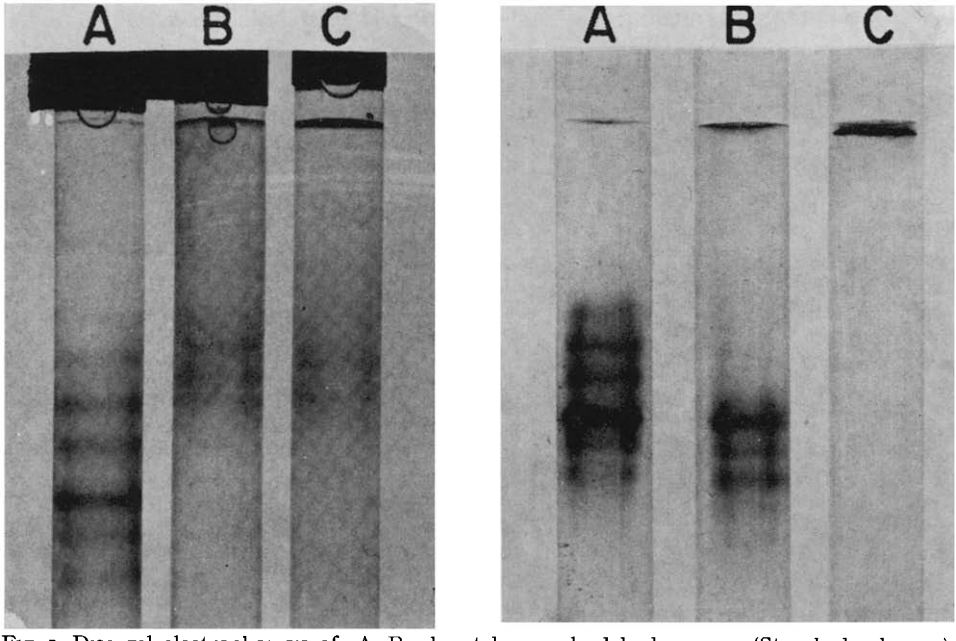

Fig 1. Disc gel electrophoresis of A Pig heart lipoamide dehydrogenase (Straub diaphorase), 20-μl sample in 0.025 M phosphate buffer (pH 7 5) (enzyme activity, 3 μmoles NADH oxidized per min) B and C Lipoamide dehydrogenase derived from pig heart α-ketoglutarate dehydrogenase Samples were electrophoresed at 1 5 mA/tube for 3 h, moving toward the anode.

Fig. 2 Disc gel electrophoresis of A Straub diaphorase B Lipoamide dehydrogenase derived from pig heart pyruvate dehydrogenase C Unresolved pig heart pyruvate dehydrogenase Conditions are the same as described in Fig 1 except for variation noted in the text.

pattern shown in Fig 1A. Six major, diaphorase-active areas are resolved which correspond to protein areas when stained with Coomassie blue and exhibit fluorescence when viewed under ultraviolet light. No other protein is demonstrable The reactive

TABLE I

DISTRIBUTION OF ENZYME ACTIVITY AFTER POLYACRYLAMIDE GEL ELECTROPHORESIS OF PIG HEART LIPOAMIDE DEHYDROGENASE

Sections were cut and eluted after electrophoresis for 3 h at 1 5 mA/tube of 20 μl enzyme solution (3 05 μmoles NADH oxidized per min per 0 01 mg)

Section No	*Distance from top (cm)*	*Tetrazolium activity*	*NADH–lipoamide reductase activity (μmoles/min)*
1	2 3	++	0 1115
2	2 7	+++	0 360
3	3 1	+++	0 405
4	3 6	++++	0 505
5	3 9	+	0 189
6	4.2	+	0 108

bands retain the same mobilities after they are sectioned and reelectrophoresed under the same conditions Resolution and number of bands are unaffected by addition of 1 mM $CaCl_2$, 1 mM dithiothreitol, or 6 M urea to the enzyme protein before electrophoresis. Neither are they altered by replacing phosphate ions in the solution by Tris buffer, or by electrophoresing samples in 0.005 M Tris–HCl (pH 8 5)–0 001 M EDTA The pattern is unchanged when gel polymerization is catalyzed by riboflavin-mediated photopolymerization instead of ammonium persulfate[15]. The number of major reactive forms observed is in agreement with earlier reported results[4,5]

That these diaphorase-active fractions are lipoamide dehydrogenase reactive is shown in Table I. Correspondence is observed between diaphorase-active areas and the enzymically catalyzed reduction of NADH in the presence of lipoamide

Figs 1B and 1C show patterns observed when lipoamide dehydrogenase derived from α-ketoglutarate dehydrogenase is similarly electrophoresed Two bands are clearly discernible, with some indication of a minor third band corresponding to the slower components of Straub diaphorase (Fig 1A)

Fig 2 shows the results of electrophoresis of lipoamide dehydrogenase obtained by urea resolution of pyruvate dehydrogenase The enzyme shows 3 main diaphorase and lipoamide dehydrogenase-active components (Fig. 2B) corresponding to the higher mobility fractions of Straub diaphorase (Fig. 2A) These gels were overstained emphasizing the absence of any enzymically reactive material in the upper portion of the gel Slight diaphorase reactive material is demonstrable at the origin and in a faster fourth band. Normally, these are not observed Unresolved pyruvate dehydrogenase (Fig 2C) fails to penetrate the gel and the area corresponding to lipoamide dehydrogenase is negative When the lipoamide dehydrogenases derived from the two α-keto acid dehydrogenases are mixed, a five-banded pattern is obtained

PETTIT AND REED[16] demonstrated electrophoretic homogeneity of the corresponding enzymes isolated from *E coli*, each being shown to exist as a single identical electrophoretic species. These findings have been confirmed under conditions in which the pig heart enzymes exhibit heterogeneity. The mobility of the *E coli* lipoamide dehydrogenase is markedly higher at pH 8 5 than that of the pig heart enzyme These electrophoretic differences further emphasize the molecular dissimilarity of the *E coli* and pig heart enzymes[1,17].

The possibility of systematic gel artifacts or artifacts arising in preparation of the enzyme cannot yet be eliminated, but when considered in conjunction with previous studies[4,5], it appears improbable that the observed patterns may be explained on that basis. If the multiple forms are not the result of differences in primary structure[18], then the importance of conformational differences in the forms must be evaluated Of direct relevance is the failure to detect immunochemical heterogeneity of the pig heart enzymes using Ouchterlony double-diffusion techniques[19,20] These aspects, together with study of the electrophoretic characteristics of free lipoamide dehydrogenase[3] are under investigation.

Department of Biochemistry and Nutrition,
Graduate School of Public Health,
University of Pittsburgh,
Pittsburgh, Pa. (U.S.A.)

M L COHN
L WANG
W SCOUTEN
I. ROSABELLE MCMANUS

1 L J REED AND D. J COX, *Ann Rev Biochem*, 35 (1966) 57.
2 U. HENNING, *Angew. Chem. Intern Ed Engl*, 5 (1966) 785
3 C J. LUSTY AND T P SINGER, *J Biol Chem*, 239 (1964) 3733
4 M R ATKINSON, R DIXON AND J M THORNBER, *Biochem J*, 82 (1962) 29P
5 A M STEIN AND J. H STEIN, *Biochemistry*, 4 (1965) 1491
6 C J LUSTY, *J Biol Chem*, 238 (1963) 3443
7 T HAYAKAWA, M HIRASHIMA, S IDE, M HAMADA, K OKABE AND M KOIKE, *J. Biol Chem.*, 241 (1966) 4694
8 M HIRASHIMA, T HAYAKAWA AND M KOIKE, *J Biol Chem*, 242 (1967) 902
9 L J REED AND C R WILLMS, *Methods Enzymol*, 9 (1966) 247
10 V MASSEY, *Biochim Biophys Acta*, 37 (1960) 310, 314
11 V MASSEY, *Methods Enzymol*, 9 (1966) 272.
12 O H LOWRY, N J ROSEBROUGH, S L FARR AND R J. RANDALL, *J Biol Chem*, 193 (1951) 265
13 J T. CLARKE, *Ann N Y Acad. Sci*, 121 (1964) 428
14 A CHRAMBACH, R A REISFELD, W WYCKOFF AND J ZACCARI, *Anal Biochem*, 20 (1967) 150
15 J M BREWER, *Science*, 156 (1967) 256
16 F H PETTIT AND L J REED, *Proc Natl Acad Sci U S*, 58 (1967) 1126
17 C H WILLIAMS, G. ZANETTI, L D ARSCOTT AND J K MCALLISTER, *J Biol Chem*, 242 (1967) 5226
18 V MASSEY, T HOFMANN AND G. PALMER, *J Biol Chem*, 237 (1962) 3820
19 A M STEIN, B WOLF AND J H STEIN, *Biochemistry*, 4 (1965) 1500
20 T HAYAKAWA, T AIKAWA, K OTSUKA AND M KOIKE, *J Biochem.*, 62 (1967) 396

Received January 30th, 1968

Biochim Biophys Acta, 159 (1968) 182–185

BBA 63312

Properties of three isoenzymes of *Clostridium pasteurianum* hydrogenase

ACKRELL, ASATO AND MOWER[1,2] showed the existence of a maximum of six hydrogenase (H_2 ferredoxin oxidoreductase, EC 1.12.1.1) isoenzymes in *Clostridium pasteurianum*. Techniques such as gel filtration and density gradient centrifugation allow the estimation of molecular size of enzymes in the presence of other proteins. They are thus particularly useful in the study of hydrogenase enzymes which, because of their well known instability[3,4], have not been purified. We report here the molecular weight and the interrelation of three of the most active hydrogenase isoenzymes (α, β, and γ).

C pasteurianum cells were grown in the medium described by CARNAHAN AND CASTLE[5]. Extracts were obtained by autolysis of 1.0 g of dried cells in 10 ml of 0.1 M potassium phosphate buffer (pH 7.0)[6], followed by centrifugation at 30 000 × *g* for 30 min. The hydrogenase-containing supernatant was stored under hydrogen.

The presence of hydrogenase activity in different bacterial extracts was determined quantitatively by manometric evolution of H_2 gas according to the method of PECK AND GEST[7]. Individual hydrogenase isoenzyme species were identified by their R_F values after hydrogenase preparations (1–5 mg of protein) were subjected to electrophoresis on polyacrylamide disc gels (7.5%)[8]. Bromophenol blue was used as the zone front for R_F determinations. The site of hydrogenase activity in the gel was located by the method of ACKRELL, ASATO AND MOWER[1] in which methyl viologen is reduced in the presence of H_2 gas.

The vertical slab gel apparatus of RAYMOND[9] was used with the same buffer