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ISOENZYMIC FORMS OF NAD-LINKED GLYCEROL-3-PHOSPHATE DEHYDROGENASE FROM RABBIT BRAIN

RICHARD KORNBLUTH, PAULA S. TRACY and THOMAS P. FONDY *

Department of Biology, Syracuse University, Syracuse, NY 13210 (U.S.A.)

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Summary

Two major enzyme forms of cytosolic NAD-linked glycerol-3-phosphate dehydrogenase in rabbit brain have been purified to apparent homogeneity. One major enzyme form designated $I_{6.5}$ exhibits an iso-electric point at pH 6.5, and is indistinguishable from the major form $I_{6.5}$ found in other tissues.

The other major form, designated $I_{5.9}$, has an isoelectric point at pH 5.9, and by amino acid analysis is shown to be a true isoenzyme distinct from form $I_{6.5}$. Form $I_{5.9}$ appears to be closely related to or identical with the major enzyme characteristic of heart. Neither the brain enzyme form $I_{5.9}$ nor the major heart isoenzyme are inhibited by antiserum to the muscle enzyme. Because of the high apparent K_m for NADH, it is postulated that the brain isoenzyme $I_{5.9}$ serves to maintain glycolysis when NADH levels rise under relatively anaerobic conditions especially during fetal and neonatal development.

Introduction

Cytosolic NAD-linked glycerol-3-phosphate dehydrogenase (*sn*-glycerol-3-phosphate:NAD^+ 2-oxidoreductase, EC 1.1.1.8) catalyzes an important metabolic link between glycolysis and phosphoglyceride pathways, and is involved in a number of distinct biochemical functions. The diversity of these functions has been documented previously [1] and includes: generation of glycerol 3-phosphate for phospholipid and triglyceride production, maintenance of cytosolic NAD^+ levels during anaerobic glycolysis, fixation of reducing equivalents into glycerol 3-phosphate for transport into the mitochondrion in the functioning of the glycerol 3-phosphate shuttle, and gluconeogenesis from glycerol.

This diversity of function is reflected in diversity of molecular form in which

the enzyme activity resides. Several forms of the enzyme have been observed in avian and insect tissues [2,3] and complex patterns of multiple forms have been identified in normal and neoplastic mammalian tissues [4—13]. Developmental changes in these patterns of multiple forms have been studied using both in vivo derived tissues [3,4,10,14] and cultured cells [15].

In rabbits [1,6], rats [6,16] and mice [5,9] large proportion of the enzyme activity in several tissues from adult animals appears in a single major form, which in rabbit exhibits an isoelectric point at pH 6.5 [1]. In heart muscle from all three species, a major component of enzyme activity appears in a relatively anionic form [1,6] which in rabbits exhibitis an isoelectric point at pH 6.1 [1]. In rat and mouse brain tissue the expression of two isoenzymic forms has been indicated [4,12] but immunological studies of rat and rabbit brain enzyme preparations showed a single major enzyme form similar to that found in skeletal muscle tissue [6,12].

We have isolated and characterized the major multiple forms of NAD-linked glycerol-3-*P*-dehydrogenase from rabbit brain tissue and determined the biochemical relationship of these forms to one another and to major isoenzyme forms previously observed in other rabbit tissues, particularly in skeletal muscle and heart.

Methods

Chemicals and animal tissues. Sources were as detailed previously [1]. Adipic acid dihydrazide was obtained from Eastman Organics (Rochester, NY). Brains and hearts were obtained from 8—12 week old rabbits and shipped fresh-frozen by Pel-Freez Biologicals (Rogers, Arkansas). Tissues were stored at −20°C and used within 7 days.

Enzyme assay and protein determination. The glycerol-3-phosphate dehydrogenase assay and definition of enzyme units were as previously described [1]. During isolation procedures, protein concentration was determined by the method of Warburg and Christian [17]. Protein content of the purified enzymes was determined by the method of Lowry et al. [18], and by amino acid analysis.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis of native enzyme was performed at 4°C in 7% gels at pH 7.5 according to the method of Orr et al. [19], run at 2 mA/gel for 20 min followed by 3 mA/gel for an additional 1.5—2 h. Gels were stained for enzyme activity as previously described [20]. SDS gel electrophoresis of dissociated enzyme was performed as previously described [1].

Acrylamide gel electrofocusing. Flat-plate acrylamide gel electrofocusing in the pH range 3.5—10 was performed on LKB Ampholyte polyacrylamide gel plates as previously described [1]. The gels were run for 2.25 h (starting voltage 200) and the voltage was increased every 10 min for 50 min up to 1000 V. Focusing was continued for another 95 min and then the gels were stained for enzyme activity.

Narrow pH range polyacrylamide plates (pH 4.0—6.5), also obtained from LKB, were run as described for the pH 3.5—9.5 plates.

Immunoelectrophoresis and immunodiffusion. Agar gel electrophoresis was

carried out in 1% Ionagar gels containing 25 mM barbital buffer (pH 8.6). Electrophoresis was run at a constant voltage of 15 V/cm for 2.5 h on an LKB Multiphor apparatus maintained at 4—6° C. After electrophoresis, the plates were either stained for enzyme activity or used for immunodiffusion, using goat antiserum to the rabbit muscle glycerol-3-phosphate dehydrogenase, prepared as previously described [11]. After electrophoresis, the antibody well was aspirated and 0.2—0.3 ml antibody (140—150 mg protein/ml) was applied. Precipitin bands were allowed to develop at 4° C for 48—72 h. Unreacted antigen and antibody were removed by washing the gels in 0.3 M NaCl for 2—3 days with several changes followed by washing in 0.15 M NaCl for 24 h. The gels were then stained for enzyme activity.

Ouchterlony double diffusion was performed on glass slides coated with 1% Ionagar containing borate-buffered saline (pH 8.4). Commercially obtained $(NH_4)_2SO_4$ suspensions of rabbit muscle glycerol-3-phosphate dehydrogenase and purified rabbit brain glycerol-3-phosphate dehydrogenase were dialyzed against 50 mM triethanolamine acetate (pH 7.5), 1 mM EDTA, 5 mM 2-mercaptoethanol (buffer 1) before being applied to the plate. Immunoprecipitin bands were developed and stained for enzyme activity as described above. The plates were also stained for protein with 0.5% Amido Black, 5% $HgCl_2$ in 5% acetic acid, and destained in 2% acetic acid.

Amino acid analysis. Procedures used for hydrolysis and amino acid analysis were as previously described [1].

Synthesis of affinity matrix. Sepharose 4B was activated with 100 mg CNBr/ml gel [21]. Adipic acid dihydrazide was attached to the activated Sepharose 4B according to the method of Wilcheck and Miron [22]. Trinitrobenzenesulphonic acid was reacted with the adipic acid dihydrazide-Sepharose as previously described [23].

Purification of brain isoenzymes. 3 kg frozen rabbit brains were allowed to partially thaw overnight in the cold room. All operations were carried out at 4° C. The tissue was homogenized in 2 vols. buffer 1 in a Waring Blendor. After stirring for 1 h to reduce foaming, the homogenate was centrifuged at 20 000 × *g* for 1 h. The supernatant was decanted through several layers of cheesecloth and the pellet was resuspended in 1 volume buffer 1, recentrifuged, and the supernatants combined. Protein was determined in the combined supernatants after passage through a Sephadex G-25 column to remove small molecular weight material absorbing at 260 nm.

The suspension was fractionated in two steps: 0—40 and 40—70% $(NH_4)_2SO_4$ saturation. After the addition of solid $(NH_4)_2SO_4$ over 3 h, the suspension was stirred for an additional 2 h and left overnight. An aliquot of the supernatant from the 0—40% $(NH_4)_2SO_4$ treatment was desalted on a Sephadex G-25 column prior to assaying in order to remove inhibitory ammonium-sulfate.

The 70% $(NH_4)_2SO_4$ precipitate was dissolved in buffer 1 and applied to three separate 2.5 × 10 cm columns of trinitrophenyl-adipic acid dihydrazide-Sepharose 4B run simultaneously. The columns were washed with 5 bed vols. buffer 1, 30% (v/v) glycerol, followed by two bed vols. buffer 1, 30%glycerol, 0.3 M KCl, and finally 6 bed vols. buffer 1, 30% glycerol. The final absorbance at 280 nm of the eluate was less than 0.02. The enzyme was eluted with 0.3 mM NADH in buffer 1, 30% glycerol.

Fractions containing enzyme activity were pooled, concentrated, and desalted on a Sephadex G-25 column equilibrated with buffer 1. The desalted enzyme solution was concentrated to 2 ml and either applied to the preparative electrofocusing column or to a 1.5 × 16 cm column of DEAE-Sephadex A-50 equilibrated in 25 mM potassium phosphate (pH 7.9), 1 mM EDTA, 5 mM 2-mercaptoethanol. The column was developed with the starting buffer until no more enzyme activity was eluted. A linear gradient was then applied consisting of 200 ml starting buffer and 200 ml 0.2 M phosphate (pH 6.6), 0.1 M NaCl, 1 mM EDTA, 5 mM 2-mercaptoethanol. Preparative electrofocusing was conducted as described previously [1] with prefocusing for either 16 or 72 h. Extended prefocusing caused the pH gradient to shift towards the cathode and towards the higher concentration of sucrose in the gradient, which resulted in a greater stabilization of the protein band containing the enzyme during elution. After focusing was complete, the enzyme was eluted, concentrated and dialyzed against buffer 1.

Partial purification of heart NAD-linked glycerol-3-phosphate dehydrogenase. Frozen rabbit hearts were allowed to thaw in 3 vols. buffer 1, cut into pieces with scissors and homogenized for 3 1-min intervals at high speed in a Waring Blendor. The homogenate was centrifuged for 1 h at 20 000 × *g*. The supernatant was poured through several layers of cheesecloth and then applied to a 2.5 × 10 cm column of trinitrophenyl-adipic acid dihydrazide-Sepharose 4B. The column was washed with 5 bed vols. buffer 1, 30% glycerol; one bed vol. buffer 1, 30% glycerol, 0.3 M KCl, and finally 5 bed vols. buffer 1, 30% glycerol. The enzyme was eluted with 500 ml buffer 1, 30% glycerol, 0.3 mM NADH. The eluate was concentrated by ultrafiltration and desalted on a Sephadex G-25 column equilibrated in buffer 1.

Results

Isolation of isoenzymically pure forms

Purification results for the two major forms of glycerol-3-phosphate dehydrogenase from rabbit brain are summarized in Tables I and II. When the mixture of enzyme forms obtained by elution from the affinity column was subjected to DEAE-Sephadex column chromatography by a slight modification of procedures previously employed for the heart enzyme [1], two zones of activity were eluted by the starting buffer wash, before the gradient was begun. The first zone was eluted by 5 column volumes, the second began to elute at 8 column volumes, and required 21 vols. for complete elution. No activity was eluted by the gradient. Employing nomenclature used in our earlier work these zones were designated IA and IB indicating that they eluted before the gradient. When the mixture of enzyme forms obtained from the affinity column was subjected directly to preparative electrofocusing, two major zones of activity were resolved (Fig. 1A). These forms are identified by their respective apparent isoelectric points. The major form designated $I_{6.5}$ obtained as shown in Fig. 1A was apparently free from contamination with the other enzyme forms when examined by analytical gel electrofocusing and was subjected to determination of protein purity and to biochemical characterization. Enzyme activity with an p*I* of 5.9 was included in a discrete peak and in a

TABLE I

PURIFICATION OF FORM $I_{6.5}$ RABBIT BRAIN GLYCEROL-3-PHOSPHATE DEHYDROGENASE

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (-fold)	Yield overall (%)
20 000 × *g* supernatant	$77.1 \cdot 10^3$	2300 *	0.03		100
40% $(NH_4)_2SO_4$ supernatant	$33.2 \cdot 10^3$	2000	0 06	2	88
70% $(NH_4)_2SO_4$ pellet	$22.5 \cdot 10^3$	2000	0.09	3	88
Affinity chromatography	15.6	1200	77.0	2600	52
Preparative isoelectric-focusing (pH 5—7):					
form $I_{6.5}$	1.8	315 *	175	5800	14
form $I_{5.9A}$	1.9	262 *	138	4600	11
form $I_{5.9B}$	1.7	151 *	89	3000	6

* Enzyme assayed at 0.5 mM dihydroxyacetone phosphate.

trailing zone. Activity in the peak and in the trailing zone was kept separate and designated $I_{5.9A}$ and $I_{5.9B}$, respectively. Comparison of $I_{5.9A}$ with $I_{5.9B}$ by gel electrophoresis and electrofocusing, and by amino acid analysis showed them to be indistinguishable. The major form designated $I_{5.9}$ could not be obtained isoenzymically pure by preparative electrofocusing of the affinity column eluate. Form $I_{5.9}$ was obtained essentially free from the other enzyme forms by prior fractionation from form $I_{6.5}$ on DEAE-Sephadex followed by

TABLE II

PURIFICATION OF FORM $I_{5.9}$ RABBIT BRAIN GLYCEROL-3-PHOSPHATE DEHYDROGENASE

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (-fold)	Yield overall (%)
20 000 × *g* supernatant	$50 \cdot 10^3$	1500 *	0.03		100
40% $(NH_4)_2SO_4$ supernatant	$28 \cdot 10^3$	1900	0.07	2.3	125
70% $(NH_4)_2SO_4$ pellet	$16 \cdot 10^3$	1400	0.09	3	93
Affinity chromatography	92	1200	13	433	80
DEAE-Sephadex:					
Zone I_A	3.8	244	65	2200	16
Zone I_B	3.8	440	117	3900	29
		616 *	162	5400	41
Preparative isoelectric-focusing (pH 5—7):					
Form $I_{6.5}$		15			1
Form $I_{5.9}$	1.0	162 *	162	5400	11

* Enzyme assayed at 0.5 mM dihydroxyacetone phosphate.

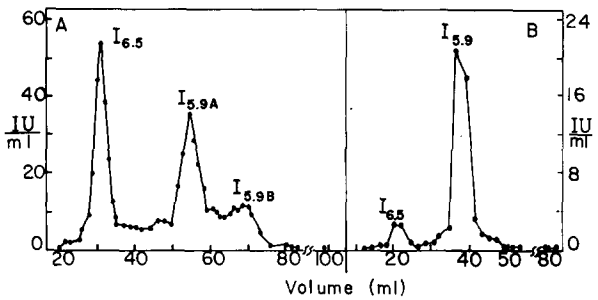

Fig. 1. Preparative 110-ml column electrofocusing (pH 5—7). A. Affinity column purified rabbit brain enzyme forms. Prefocused for 16 h; focused for 36 h after sample introduction. B. DEAE-Sephadex column purified rabbit brain enzyme zone IB. Prefocused for 72 h; focused for 36 h.

preparative electrofocusing (Fig. 1B). In two additional enzyme purifications not detailed here the apparent p*I* values obtained for the anionic brain form were 6.1 and 6.0.

Flat plate polyacrylamide gel electrofocusing

Affinity column purified enzyme showed two major zones of activity when examined by analytical flat plate gel electrofocusing pH 3.5—10 (Fig. 2A). In some preparations the two zones exhibited approximately equal enzyme staining intensity while in other preparations the cationic zone stained more intensely than the anionic zone. Analytical flat plate electrofocusing of the purified forms $I_{6.5}$ and $I_{5.9}$ showed them to be isoenzymically homogeneous and to correspond to the two major zones observed prior to preparative column

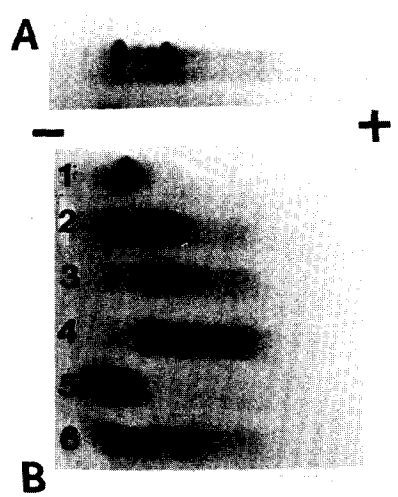

Fig. 2. Flat plate polyacrylamide gel electrofocusing pH 3.5—10. Stained for enzyme activity. A. Brain enzyme forms affinity column purified, before preparative column electrofocusing, 0.3 IU. B. Enzyme forms isolated by preparative column electrofocusing: 1. Brain enzyme form $I_{6.5}$, 0.4 IU. 2. Brain enzyme activity between the major peaks in Fig. 1, showing presence of form $I_{6.3}$, 0.4 IU. 3. Brain enzyme form $I_{5.9A}$, Fig. 1, 0.4 IU. 4. Brain enzyme activity from zone $I_{5.9B}$, Fig. 1, 0.3 IU. 5. Brain enzyme form $I_{6.5}$ + muscle enzyme. 6. Brain enzyme form $I_{6.5}$ + brain enzyme form $I_{5.9}$.

electrofocusing (Fig. 2B, tracks 1—4). Form $I_{6.5}$ did not resolve from the major rabbit muscle enzyme form designated $I_{6.5}$ in our previous work (Fig. 2B, track 5).

Polyacrylamide gel electrophoresis

SDS gel electrophoresis of the isoenzymically pure form $I_{6.5}$ at 50 μg protein per gel showed the presence of a single zone staining for protein. Absence of measureable protein contamination for isoenzymically pure form $I_{5.9}$ was established at two protein concentrations (Fig. 3A). Polyacrylamide gel electrophoresis of the active enzyme forms using the pH 7.5 system of Orr et al. [19] showed that brain enzyme form $I_{6.5}$ migrated identically with the rabbit muscle enzyme when they were run separately and in mixtures, but was resolved from form $I_{5.9}$ (Fig. 3B).

Heat inactivation

Heat inactivation was performed on the affinity column eluate and on the purified and isolated peaks from preparative electrofocusing after dialysis against buffer 1. At 47.5°C, the affinity column eluate was resolved into two forms: a rapidly inactivating form representing about 40% of the total enzyme activity, and a more slowly inactivating form representing about 60% of the total activity (Fig. 4A). The heat labile component of the affinity column eluate corresponded to the anionic form $I_{5.9}$, while the heat stable component

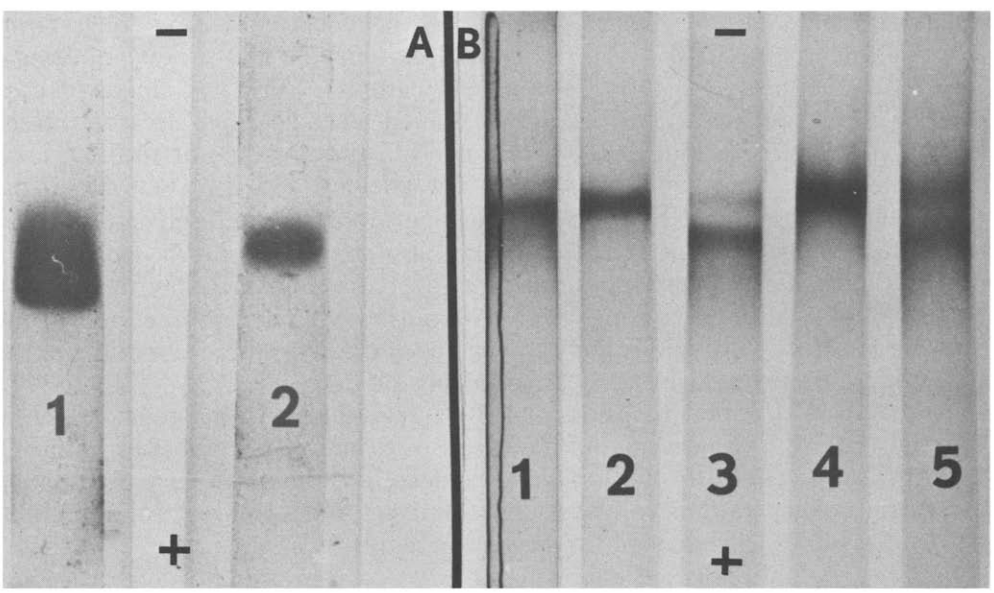

Fig. 3. Gel electrophoresis of rabbit brain and muscle isoenzymes. A. SDS polyacrylamide gels of brain enzyme form $I_{5.9}$ at 100 μg (gel 1) and 50 μg (gel 2); stained for protein. B. Polyacrylamide gels stained for enzyme activity: 1. Commercially obtained muscle enzyme (mainly form $I_{6.5}$), 0.25 IU. 2. Brain enzyme form $I_{6.5}$, 0.25 IU. 3. Brain enzyme form $I_{5.9}$, 0.25 IU. 4. Mixture of 0.25 IU each of brain enzyme form $I_{6.5}$ and commercially obtained muscle enzyme. 5. Mixture of 0.25 IU each of brain enzyme form $I_{5.9}$ and commercially obtained muscle enzyme.

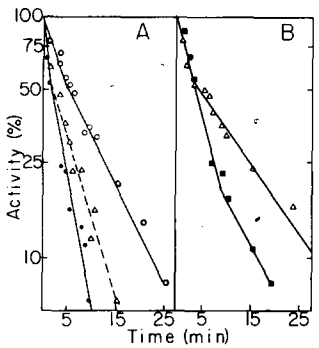

Fig. 4. A. Heat inactivation at 47.5° of brain isoenzyme forms: (△- - - - -△) mixed isoenzyme forms from affinity chromatography; (○———○) purified form $I_{6.5}$; (●———●) purified form $I_{5.9}$. B. Heat inactivation at 46° of brain and heart isoenzyme forms: mixed brain (△———△) and heart (■———■) isoenzyme forms from affinity chromatography.

corresponded to the cationic form $I_{6.5}$. When affinity column-purified heart and brain glycerol-3-phosphate dehydrogenases were analyzed by heat inactivation at 46°C (Fig. 4B), the heat-labile form from heart inactivated at the same rate as form $I_{5.9}$ from brain.

Immunochemical studies

Pure form $I_{6.5}$ and partially purified form $I_{5.9}$ from preparative electrofocusing were subjected to immunoelectrophoresis along with commercially-obtained rabbit muscle glycerol-3-phosphate dehydrogenase (Fig. 5A). Form $I_{6.5}$ coelectrophoresed with the rabbit muscle enzyme and produced a single precipitin arc located slightly towards the cathode. Form $I_{5.9}$ migrated as a discrete zone towards the anode when stained after agar gel electrophoresis. Only a very faint precipitin arc was obtained in the presence of antibody, but could be easily visualized by staining for enzyme activity (Fig. 5B). This precipitin arc produced by form $I_{5.9}$ and anti-rabbit muscle glycerol-3-phosphate dehydrogenase antibody was very close to the antibody trough indicating a large excess of cross-reacting antigen. This could possibly be due to a slight contamination of the antiserum with antibody to the anionic form of the enzyme (form $I_{5.9}$) which is likely to have been present in trace amounts in the rabbit muscle enzyme used as the immunizing antigen.

Ouchterlony double diffusion plates of rabbit muscle enzyme and brain enzyme form $I_{6.5}$ showed a continuous arc with no spurs, indicating antigenic identity. No precipitin formation could be detected when form $I_{5.9}$ was applied at several concentrations to plates containing serial dilutions of the antibody, even when plates were stained for enzyme activity.

Affinity column-purified rabbit brain glycerol-3-phosphate dehydrogenase containing both forms $I_{6.5}$ and $I_{5.9}$ was less than 50% inhibited by antibody to the rabbit muscle enzyme under conditions where the rabbit muscle enzyme was totally inhibited (Fig. 6). The uninhibited portion of the rabbit brain enzyme represented about the same proportion of the total activity in brains as the heat labile form determined by heat inactivation.

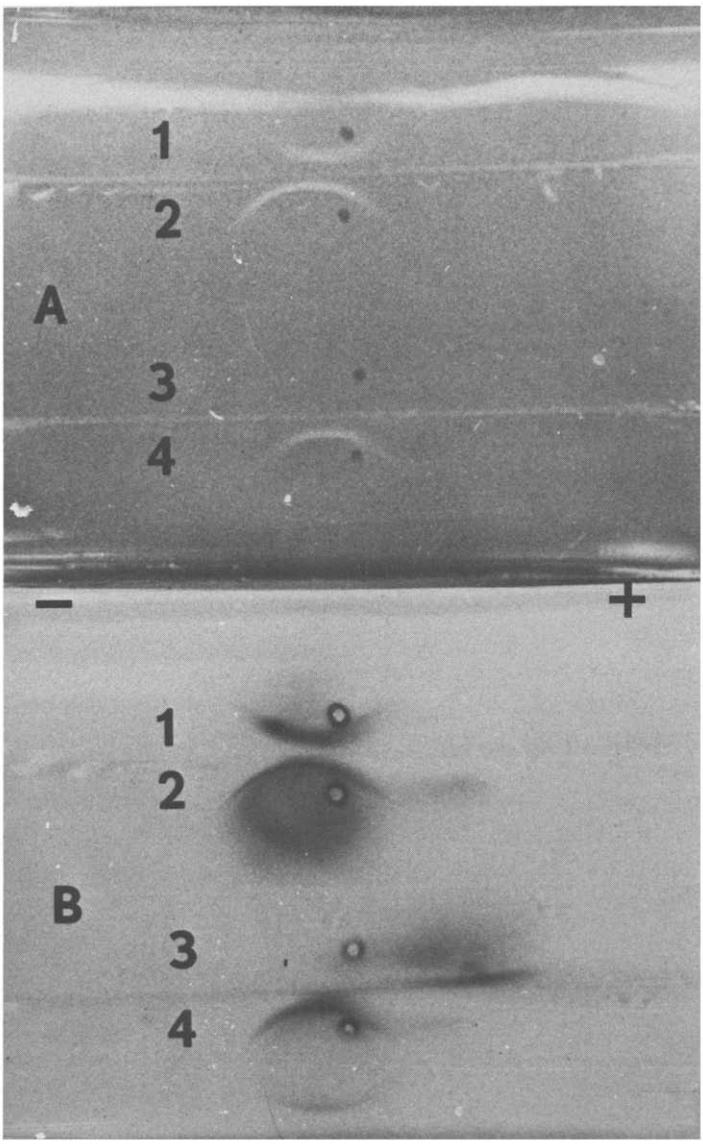

Fig. 5. Immunoelectrophoresis of brain and muscle isoenzymes. 1. Commercially obtained muscle enzyme (mainly form $I_{6.5}$), 0.14 IU. 2. Brain form $I_{6.5}$, 0.14 IU. 3. Brain form $I_{5.9}$, 0.14 IU. Mixture of 0.14 IU each of brain form $I_{6.5}$ and commercially obtained muscle enzyme. A. Unstained precipitin arcs. B. Stained for enzyme activity.

Subunit molecular weight

The subunit molecular weight of the brain enzyme was determined by polyacrylamide gel electrophoresis in the presence of 0.1% SDS. The subunit molecular weight standards used were pyruvate kinase (57 000), glutamic dehydrogenase (53 000), ovalbumin (43 000), aldolase (40 000), glyceraldehyde-3-*P* dehydrogenase (36 000), carbonic anhydrase (29 000), chymotrypsinogen (25 700) and trypsin (23 300). Rabbit brain glycerol-3-phosphate dehydro-

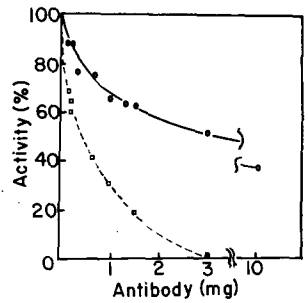

Fig. 6. Inhibition of activity of brain and muscle enzymes with goat anti-serum to commercially obtained rabbit muscle enzyme. (○———○) Affinity column purified brain isoenzyme forms. (□- - - - -□) Muscle enzyme. Activity after incubation for 16 h, 4°C, 0.8 IU, 1.0 ml total volume, followed by centrifugation and assay of supernatant. Control enzyme incubated in absence of antibody.

genase forms $I_{6.5}$ and $I_{5.9}$ each produced a single band corresponding to a subunit molecular weight of 37 000, which is in close agreement to the subunit molecular weight of the rabbit muscle enzyme as determined by amino acid analysis.

Kinetics: apparent K_m values

The apparent K_m values for the four substrates are shown in Table III along with comparison of K_m values obtained previously for potentially corresponding isoenzyme forms from rabbit muscle and heart. In repeat determinations of these values, results did not differ by more than 20% from the results shown in Table III. The apparent K_m values for form $I_{6.5}$ from rabbit brain for all four substrates are indistinguishable from those obtained for form $I_{6.5}$ isolated from muscle, mammary gland, liver, and adipose tissue. The apparent

TABLE III

APPARENT MICHAELIS CONSTANTS FOR RABBIT BRAIN GLYCEROL-3-PHOSPHATE DEHYDROGENASE

Assays in the direction of dihydroxyacetone-*P* reduction were in 0.05 M Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM 2-mercaptoethanol. Assays in the direction of glycerol-3-*P* oxidation were in 0.05 M pyrophosphate (pH 9.0), 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.1 M hydrazine. All assays were done at room temperature. Values shown are means of triplicate determinations. Ranges did not exceed ± 25%.

Substrate varied	Cosubstrate	Concentration (mM)	Apparent K_m (mM)			
			Form $I_{6.5}$	Form $I_{5.9}$	Muscle * Form $I_{6.5}$	Heart * Form $II_{6.1}$
Dihydroxyacetone-*P*	NADH	0.1	0.2	0.05	0.22	0.20 **
NADH	Dihydroxyacetone-*P*	0.5	0.01	0.15	0.008	0.10
DL-Glycerol-3-*P*	NAD^+	0.3	0.3	0.35	0.18	0.17
NAD^+	DL-Glycerol-3-*P*	10.0	0.01	0.05	0.012	0.06

* Taken from Ostro and Fondy [1].

** At saturating NADH (5 mM).

K_m values exhibited by form $I_{5.9}$ for NADH, DL-glycerol-3-*P*, and NAD^+ are not significantly different from corresponding values obtained for form $II_{6.1}$ from heart, but are clearly distinct from the values exhibited by form $I_{6.5}$. The apparent K_m value for dihydroxyacetone-*P* obtained with form $I_{5.9}$ from brain differs by 4-fold from the value obtained with form $II_{6.1}$ from heart, but the cosubstrate (NADH) concentration was saturating in the case of the heart form and nonsaturating in the case of the brain form rendering the apparent difference insignificant.

Amino acid analysis

Table IV shows the amino acid composition of brain enzyme forms $I_{6.5}$ and $I_{5.9}$ and their comparison with the potentially corresponding forms $I_{6.5}$ from skeletal muscle, and $II_{6.1}$ from heart. Since there are no significant differences in the values for any of the amino acid residues in comparing muscle enzyme form $I_{6.5}$ with brain enzyme form $I_{6.5}$, it is apparent that the major form $I_{6.5}$ from brain corresponds with the similar major form from skeletal muscle and thus with the common major form from mammary gland, liver, and adipose tissue [1,11]. Brain enzyme form $I_{5.9}$ on the other hand is clearly distinct from form $I_{6.5}$ in the values obtained for lysine, arginine, aspartic acid, threonine, serine, isoleucine, and tyrosine, all of which differ by more than 20% and some by as much as 100% between the two enzyme forms. Since some of these amino acid residues are present in lower integral numbers and others in higher integral numbers in one form compared to the other, the forms cannot be derived from one another by proteolysis or deamination but rather must represent distinct isoenzymic forms. Comparison of form $I_{5.9}$ from brain with form $II_{6.1}$ from heart shows them to be closely related in amino acid composition, but to differ to a maximum of 20% in the values obtained for lysine, histidine, serine, isoleucine, and tyrosine. It is not clear whether these differ-

TABLE IV

AMINO ACID COMPOSITIONS OF THE MAJOR BRAIN FORMS OF GLYCEROL-3-PHOSPHATE DEHYDROGENASE AND COMPARISON WITH MAJOR FORMS FROM HEART AND MUSCLE

Amino acid	Muscle [1] $I_{6.5}$	Brain $I_{6.5}$	Brain $I_{5.9}$	Heart [1] $II_{6.1}$
Lys	27	29	23	19
His	9	8	7	9
Arg	9	9	14	14
Asp	27	28	33	33
Thr	13	13	19	19
Ser	10	10	18	22
Glu	41	41	41	41
Pro	14	14	13	14
Gly	39	38	34	38
Ala	31	32	30	28
Val	31	31	29	30
Met	8	7	8	7
Ile	28	26	19	15
Leu	29	28	33	29
Tyr	5	5	11	13
Phe	15	15	14	14

ences are due to artifacts in hydrolysis or analysis, to low levels of the major isoenzyme form $I_{6.5}$ contaminating the preparation of brain enzyme form $I_{5.9}$, or whether a real but subtle distinction exists in amino acid composition between the anionic enzyme form heart and brain.

Comparison of brain form $I_{5.9}$ with heart form $II_{6.1}$

Form $I_{5.9}$ from brain and form $II_{6.1}$ from heart appeared to correspond to one another in their apparent K_m values (Table III), and in rates of heat inactivation (Fig. 3B). In two other preparations of the brain enzyme apparent p*I* values indistinguishable from values obtained for the heart enzyme were observed indicating that the difference in p*I* values of 5.9 compared to 6.1 is not significant. In order to test this, affinity column-purified isoenzyme forms from brain and heart were examined by analytical flat plate polyacrylamide gel electrofocusing at pH ranges of 3.5—10 and 4.0—6.5. They gave indistinguishable patterns when run in parallel tracks, and gave no evidence of resolution from one another when analyzed in mixtures. In preparations carried out separately, there was an apparent difference between the heart and brain isoenzymes in their elution from DEAE Sephadex. However, under conditions used in this present paper, DEAE Sephadex column chromatography of affinity column purified brain and heart enzyme forms whether run separately or in mixtures gave two major zones of enzyme activity corresponding to zones IA and IB observed in the purification of the brain isoenzymes, both eluting before the gradient. Thus they behaved identically and were in fact indistinguishable by this criterion.

Discussion

Affinity chromatography

The affinity matrix originally developed in our work employed hexamethylenediamine as a spacer arm for the trinitrophenyl ligand [23] and produced a 400-fold purification of the rabbit brain glycerol-3-phosphate dehydrogenase when applied on a preparative scale. However, the yield of activity was in the range of 50—60%. Some of the remaining activity was tightly bound to the gel itself since used matrix was enzymically active. The tight binding may have been due to the detergent-like effects produced by the charged isourea linkage and hydrophobic spacer arm reported by Hofstee and others [24—26]. By utilizing the charge-free dihydrazide spacer arm of Wilcheck and Miron [22], we have improved the recovery and the purity of the eluted enzyme. The recovery of bound units has been further increased by using glycerol in the wash and eluting buffers, although a loss in purification accompanied the increased yield in this case. Although the exact nature of the bio-specific interaction of the enzyme with the matrix is unknown, the binding may be similar to the interaction of several dehydrogenases with Dextran Blue [27] in which the binding appears to involve the dinucleotide fold of the coenzyme binding site. Interaction of the trinitrophenyl group with a coenzyme binding site seems likely since low concentrations of coenzyme bring about elution.

Multiple forms

Multiple forms of glycerol-3-phosphate dehydrogenase have been reported in

rat [6,12] and mouse [4] brain. In rat brain, multiple forms were detected by polyacrylamide gel electrophoresis and DEAE-cellulose ion-exchange chromatography, although antibody made to one of the electrophoretic forms was able to inhibit all of the enzyme present in the purified sample [12]. Similarly, antiserum to rat muscle enzyme totally inactivated rat brain enzyme, but it is not totally clear whether this observation extended also to rabbits [6]. In mouse brain, two forms were found which differed in their thermal stability and elution pattern on DEAE-cellulose ion-exchange chromatography and appeared to be under the control of separate genes. The two major forms of the enzyme that we found in rabbit brain appear to be distinct isoenzymes, since they differ in their isoelectric points, electrophoretic mobilities, antigenic determinants, heat stabilities, apparent K_m values for dihydroxyacetone phosphate and the coenzymes, and in amino acid composition. The more cationic form $I_{6.5}$ appears to be chemically indistinguishable from the major form of the enzyme found in skeletal muscle, mammary gland, liver, and adipose tissue. Highly sensitive catalytic studies on the other hand, have indicated that the major cationic enzyme form $I_{6.5}$ from rabbit muscle is functionally distinct from the comparable liver form $I_{6.5}$ since differential activation or inhibition by various detergents has been observed [28]. However, previous work has established that rabbit liver contains a significant proportion of glycerol-3-phosphate dehydrogenase activity in a form with an p*I* of 6.3 and thus designated $I_{6.3}$ [1]. Form $I_{6.3}$ is readily distinguishable from form $I_{6.5}$ when co-electrofocusing is carried out. Fig. 1 of ref. 28 shows that the liver enzyme preparation being characterized could contain mixed forms $I_{6.5}$ and $I_{6.3}$ since a single peak of activity appears at pH 6.4 on analytical electrofocusing. More recent work [29,30] has tended to minimize the possibility that liver form $I_{6.3}$ present at different concentrations in preparations of the liver and muscle is responsible for the important catalytic differences between the enzyme activity from rabbit liver and muscle. However, even in that work it is not clear at what point form $I_{6.3}$ has been removed from either the liver enzyme preparations or from the commercially provided rabbit muscle enzyme. In any event, it is clear from the studies of McLoughlin et al. [28—30] that rabbit liver contains glycerol-3-phosphate dehydrogenase activity that has important functional differences from the enzyme activity present in skeletal muscle. Whether liver enzyme form $I_{6.3}$ has anything to do with those differences is not known, but it is known that form $I_{6.3}$ exhibits high apparent K_m values for both NAD^+ and glycerol 3-phosphate [1].

Nature and role of the brain enzyme form $I_{5.9}$

The anionic brain enzyme form $I_{5.9}$ appears to be structurally and functionally very similar to or indistinguishable from the anionic heart form $II_{6.1}$ detailed in our earlier work [1]. Based on its kinetic properties the heart enzyme form $II_{6.1}$ was postulated to be important in maintaining high glycolytic activity by catalyzing the generation of NAD^+, when NADH concentration became elevated. A similar role for this enzyme form can be visualized in brain. Studies in mouse brain have shown a progressive shift from an anionic heat-labile enzyme form to a cationic heat-stable form during development [4]. If heat-labile form $I_{5.9}$ from rabbit brain is functionally and developmentally

correlated with the heat-labile mouse brain enzyme form, then it can be postulated that rabbit brain enzyme form $I_{5,9}$ functions to provide small amounts of NAD^+ necessary to maintain glycolysis during fetal and neonatal development. This function is supported by evidence that fetal and neonatal brain is much less dependent on aerobic respiration than is adult brain, and is capable of sustaining anaerobic glycolysis.

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