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Rapid Preparation of Mitochondrial Malate Dehydrogenase from Rat Liver and Heart†

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ABSTRACT: Mitochondrial malate dehydrogenase (L-malate:NAD^+ oxidoreductase, EC 1.1.1.37) has been purified from both rat liver and rat heart by using Sepharose–Blue Dextran pseudoaffinity chromatography. Under the conditions employed most of the mitochondrial malate dehydrogenase and some cytosolic malate dehydrogenase are adsorbed, whereas many proteins simply pass through. The blue column is then subjected to several treatments which elute a variety of other proteins (notably residual cytosolic malate dehydrogenase and lactate dehydrogenase) which also have an affinity for the bound dye Cibacron Blue F3GA. Mitochondrial malate dehydrogenase is then eluted by forming an abortive ternary complex with reduced nicotinamide adenine dinucleotide–sodium D(+)-malate. Pooled active fractions are passed through a preequilibrated diethylaminoethyl–Sephadex column which retains contaminating proteins. The active fractions are concentrated, and residual contaminants are removed by very small stepwise pH changes on a CM-52 cellulose column. The procedure is mild and rapid and yields enzyme which is homogeneous by the applied criteria. In addition, lactate dehydrogenase may be copurified. The purified mitochondrial malate dehydrogenases from liver and heart have been characterized and compared. Both enzymes show evidence of multiple forms upon starch or polyacrylamide gel electrophoresis or upon isoelectric focusing. These forms are not generated artifactually during purification since they correspond to forms seen in crude extracts. The heart enzyme contains more high p*I* forms than the liver enzyme. Sodium dodecyl sulfate electrophoresis indicated identical subunit molecular weights of 35 000 for enzyme from both sources. Ultraviolet spectra were practically identical. The mild isolation procedure produces mitochondrial malate dehydrogenase of high (if not maximal) specific catalytic activity. Amino acid analyses of heart and liver mitochondrial malate dehydrogenase showed almost identical values, and the high levels of amide nitrogen are consistent with the high p*I* values (9.0–9.5) obtained by column electrofocusing. In addition, the procedure described produces enzymes with small, somewhat variable amounts of tightly complexed glycerophospholipids.

Mitochondrial malate dehydrogenase (m-MDH)[1] has been isolated and partially characterized from various sources (Ochoa, 1955; Wolfe & Neilands, 1956; Davies & Kun, 1957; Thorne, 1960; Grimm & Doherty, 1961; Sophianopoulos & Vestling, 1962; Fahien & Strmecki, 1969; Glatthaar et al., 1974). The isolation procedures have been time consuming and the yields rather low. In the case of rat liver m-MDH, difficulties have been encountered with respect to the stability of the purified enzyme and with respect to limited proteolysis and/or deamidation which led to the apparent modification of one of the two subunits but to no loss of maximum specific activity (Mann & Vestling, 1968, 1969, 1970).

We have developed an efficient rapid isolation procedure for m-MDH using Sepharose–Blue Dextran affinity chromatography (Ryan & Vestling, 1974). We have also reexamined our evidence for the existence of nonidentical subunits in the case of rat liver m-MDH. If care is taken to avoid limited proteolysis and/or deamidation, only one subunit is produced in the procedure to be described. This result is noted under subunit dissociating conditions during polyacrylamide

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[1] Abbreviations used: m-MDH, mitochondrial malate dehydrogenase; NAD^+, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; CM, carboxymethyl; U, international MDH units; $NaDodSO_4$, sodium dodecyl sulfate.

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gel electrophoresis in 0.9 N acetic acid–6.25 M urea, pH 3.2, or $NaDodSO_4$–polyacrylamide gel electrophoresis in 0.3 M Tris-HCl, pH 8.9. However, starch gel electrophoresis in 5 mM sodium citrate, pH 5.4, consistently reveals the presence of one major cathodic component, as shown by activity staining, and smaller amounts of one or more slower migrating components. Polyacrylamide electrophoresis has also been employed to show the heterogeneity of purified heart and liver m-MDH. Since we have purified this enzyme under extremely mild conditions with the inclusion of protease inhibitor and since the observed heterogeneity parallels that seen in crude homogenates, we do not believe that the multiplicity is an artifact of purification (Glatthaar et al., 1974).

Another purpose of this paper is to reexamine the issue of mitochondrial malate dehydrogenase multiplicity (Kitto et al., 1966). We have shown that rat liver and heart m-MDHs are extremely similar, if not identical, with respect to their subunit composition, spectral properties, and amino acid composition. The difference in apparent isoelectric points that we report may reflect the existence of enzyme species caused by differential protein–phospholipid interaction (Jones & Vestling, 1978a,b).

Material and Methods

Materials. Rat livers and hearts were removed from exsanguinated animals, immediately frozen on dry ice, and stored at −20 °C till required. Blue Dextran and DEAE-Sephadex A-50 were purchased from Pharmacia Fine Chemicals, Inc. NAD^+, NADH, sodium oxamate, L(−)-malic acid, D(+)-malic acid, phenylmethanesulfonyl fluoride, and Sepharose 4B were purchased from Sigma Chemical Co. 2-Mercaptoethanol, cyanogen bromide, *N,N'*-methylenebis(acrylamide), and *N,N,N',N'*-tetraethylmethylenediamine were obtained from Eastman Kodak Co. Urea and hydrolyzed starch from Connaught Medical Research Laboratories were supplied by Fisher Scientific Co. Nitroblue tetrazolium chloride and phenazine methosulfate were obtained from Aldrich Chemical Co. Ampholines for electrofocusing were obtained from LKB. Other chemicals were of standard reagent grade quality. 2-Mercaptoethanol was redistilled before use.

Dry DEAE-Sephadex A-50 (8 g) was soaked in a total of 800 mL of 10 mM Tris, pH 8.6, for about 8 h, the buffer being changed every hour. The slurry was poured into a 4.0-cm column to a bed height of 20 cm and washed with 2 L of the same buffer.

CM-52 cellulose was precycled with NaOH and HCl according to the instructions supplied by Whatman (W. & R. Balston, Ltd., England).

Coupling of Blue Dextran to Sepharose 4B. Blue Dextran was coupled to Sepharose 4B by the cyanogen bromide procedure (Cuatrecasas, 1970) as described by Ryan & Vestling (1974) but with the use of 2 times as much cyanogen bromide. It was determined that 0.5 g of Blue Dextran was covalently attached to 50 g of Sepharose 4B (Chambers, 1977).

Protein Concentration Determination. The protein concentration of crude extracts was determined by the biuret method using bovine serum albumin as a standard (Bardawill et al., 1949). The concentration of purified m-MDH was calculated from the absorbance coefficient at 280 nm ($E_{1cm}^{1\%}$ = 2.52) as determined from the dry weight of pure enzyme. m-MDH was assayed by measuring the rate of NAD^+ reduction at 340 nm as described by Sophianopoulos & Vestling (1962).

Electrophoresis. Starch gel (14%) was obtained by dispersing hydrolyzed starch in 10 mM sodium citrate buffer, pH 5.8, in 21 × 8.3 × 1 cm Lucite trays. Electrophoresis was carried out at 2–4 °C for 18–20 h at 140 V and 30 mA with 30 mM sodium citrate, pH 5.4, in the electrode chambers. At the end of electrophoresis, the gel was sliced longitudinally into two or three slabs and stained for MDH activity as described by Fine & Costello (1963).

Polyacrylamide gel electrophoresis was performed according to Panyim & Chalkley (1969) using 7.5% polyacrylamide gel prepared in 0.8 M acetic acid–6.25 M urea, pH 3.2, or according to Davis (1964) with the use of 8% polyacrylamide gel: 0.3 M Tris–HCl, pH 8.9, in gel; 0.005 M Tris–0.038 M glycine, pH 8.3, in electrode compartments. In both methods Coomassie Blue R was used for protein staining. The procedure of Dietz & Lubrano (1967) was adapted for activity staining.

$NaDodSO_4$–polyacrylamide denaturing electrophoresis was performed in vertical slab gels (12% running gel and 4% stacking gel) according to the method originally described by Laemmli (1970) as modified by O'Farrell (1975).

Column Isoelectric Focusing. Column electrofocusing in the range pH 9–11 was performed in a sorbitol-stabilized gradient with an LKB electrofocusing column of volume 110 mL. The gradient was mixed according to the instructions enclosed with the pH 9–11 ampholines. Focusing was complete after 40 h. The column was emptied via a peristalic pump, and the A_{280} was continuously monitored while 1.5–1.7-mL fractions were collected. Fractions were assayed for pH, MDH activity, and A_{280}.

Amino Acid Analysis. Purified samples of liver and heart m-MDH were dialyzed with several changes against distilled water. (A sample of the final dialysis water served as a control.) Samples were placed in appropriately washed thick-walled Pyrex tubes and brought to dryness under partial vacuum. Two milliliters of 6 N hydrochloric acid was added to the protein, and the tubes were sealed under vacuum. Hydrolysis was allowed to take place for 22, 46, and 72 h at 110 or 138 °C. The hydrolysate was brought to dryness and the mixture of amino acids dissolved in the sample buffer. Amino acid analysis was performed with a Beckman 120 C amino acid analyzer. Results were calculated by comparison with the color values of known standards and were expressed as residues per subunit of enzyme (35 000 daltons). Control samples contained some ammonia, and this was subtracted from the tests before calculations were made. This procedure represents an adaptation of the original methodology of Moore & Stein (1963).

Enzyme Purification. m-MDH was purified from 150 g of pooled rat hearts or livers by using a new rapid procedure. The tissue was homogenized in 8 volumes (i.e., 8 × 150 mL) of 20 mM Tris-HCl, pH 8.6, containing 1 mM 2-mercaptoethanol and 1 mM phenylmethanesulfonyl fluoride (PMSF) to inhibit proteolysis which may cause experimental artifacts (Cassman & King, 1972).[2] The extract was centrifuged at 4 °C for 30 min, the supernatant was saved, and the pellet was resuspended in 3 volumes of extracting buffer, reextracted, and centrifuged. Combined supernatants were applied to a Sepharose–Blue Dextran column (4.5 × 20 cm) preequilibrated with homogenizing buffer. The column was washed with homogenizing buffer until the A_{280} of the effluent fell to less than 0.05. The column was then treated serially with (1) 2 volumes of 1 mM NAD^+–1 mM sodium L-malate, (2) 4 volumes of 10 mM Tris-HCl buffer, pH 8.6, (3) 3 volumes of 1 mM NADH in 10 mM Tris-HCl, pH 8.6, (4) 5 volumes

[2] All buffers described in the purification procedure contained 1 mM redistilled 2-mercaptoethanol.

Table I: Summary of Purification Procedure

fraction	vol (mL)	U/mL	sp act. (U/mg of protein)	% recovery
20 mM Tris extraction				
liver	1244	4.06	0.13	100
heart	1200	9.25	0.68	100
NADH-D(+)-malate eluate				
liver	420	5.37	*a*	43.6
heart	500	10.67	*a*	48.0
concentrated DEAE-Sephadex A-50 eluate				
liver	40	57.07	*a*	45.2
heart	40	152.50	*a*	55.0
concentrated CM-52 column fraction (pH 5.55)				
liver	12	160.80	111.2	38.0
heart	14	412.29	142.0	52.0

[a] Not determined due to presence of NADH.

of 10 mM Tris-HCl, pH 8.6, (5) 2 volumes of 1 mM NADH–8 mM sodium oxamate in 10 mM Tris-HCl, pH 8.6, and (6) 4 volumes of Tris-HCl, pH 8.6. m-MDH was eluted from the Blue Dextran–Sepharose column by treatment with 3 volumes of 5.5 mM NADH–5.5 mM sodium D(+)-malate in 10 mM Tris-HCl, pH 8.6, followed by 5 volumes of 10 mM Tris-HCl, pH 8.6. The partially purified m-MDH eluted was passed directly through a prepacked, preequilibrated (10 mM Tris-HCl, pH 8.6) DEAE-Sephadex A-50 column (4.0 × 20 cm). Contaminating proteins and some dye were bound while the enzyme was not. Active fractions were pooled, concentrated to ~20 mL in an Amicon ultrafiltration cell, diluted with 20 mL of 20 mM sodium citrate, pH 4.50, and applied to a CM-52 cellulose column (2.5 × 30 cm) equilibrated with 20 mM citrate, pH 4.5. Most contaminating proteins were eluted from the column by washing with this buffer until the A_{280} of the effluent fell to 0.005. Pure m-MDH was eluted by a stepwise gradient of 20 mM citrate, pH 5.33, followed by 20 mM citrate, pH 5.55; the activity eluted at pH 5.55. Fractions of 7.5 mL were collected. The whole procedure may be completed in 48 h. Peak fractions were concentrated to ~6 mg/mL in an Amicon ultrafiltration cell. The enzyme may then be stored frozen (−20 °C for periods up to 3 months) without appreciable loss of activity.

Enzyme from both heart and liver was judged to be pure by using the following criteria: (a) denaturing and nondenaturing polyacrylamide electrophoresis; (b) high specific catalytic activity; (c) sedimentation velocity analysis; and (d) column isoelectric focusing profile.

Results

Table I shows the results of the purification procedure. It is apparent that the enzyme has been purified about 850-fold from the liver and about 210-fold from the heart. It is quite obvious that heart is by far the better source of the enzyme. This apparently is quite generally true of the mammalian enzyme and probably explains why most interest has been focused on the heart as source material (Banaszak & Bradshaw, 1975). The apparent increase in recovery between stage 2 and stage 3 of the purification may represent removal of some endogenous inhibitor.

The key to obtaining high specific activity enzyme is found in the last step of the procedure. The elution patterns in Figure 1 indicate clearly the pH sensitivity of the binding of m-MDH to the carboxymethylated cellulose. If a reasonable family of pK_a values is assumed for the carboxymethyl groups (e.g., 3–5),

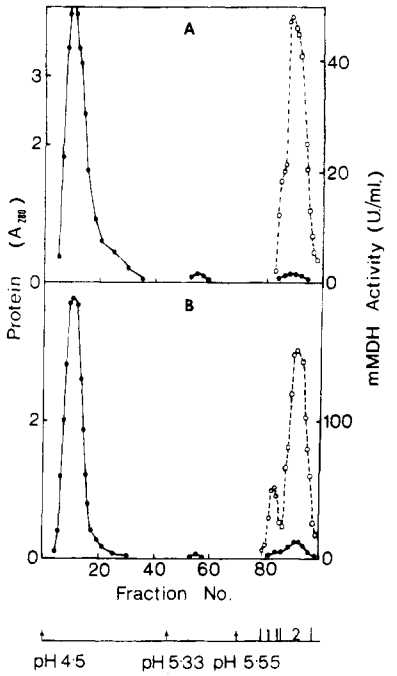

FIGURE 1: Stepwise pH elution of m-MDH from the CM-52 cellulose column. Initial buffer was 0.02 M sodium citrate, pH 4.50, second buffer was 0.02 M sodium citrate, pH 5.33, and third buffer was 0.02 M sodium citrate, pH 5.55. These buffer changes are clearly indicated by the vertical arrows. (A) Elution pattern of the liver enzyme and (B) elution pattern of the heart enzyme [(●) A_{280}; (○) MDH activity]. The smaller numbers 1 and 2 enclosed by short vertical lines indicate the fractions taken for peak 1 and peak 2 enzyme from the heart purification. 7.5-mL fractions were collected in either case.

it would appear that the main impact of changing the pH from 5.33 to 5.55 is probably directed to the protein and not to the resin. There will be only a small increase in the ionic strength of the 0.02 M sodium citrate buffer as the pH changes from 5.33 to 5.55 and the concentration of the citrate trivalent anion increases (pK_{a_2} = 4.76; pK_{a_3} = 6.40). However, this effect would not likely make a major contribution. Accordingly, one may suggest that a change in localized charge on the m-MDH takes place in the pH 5.33 to 5.55 region. Indeed, the apparent fractionation of the heteromorphs of the heart enzyme (Figure 1B) clearly supports this contention. Examination of Figure 4 in conjunction with Figure 1B shows that replacement of pH 5.33 buffer with pH 5.55 buffer results in the progressive elution of increasingly more positively charged multiple forms of activity.

The distribution of multiple forms of activity observed throughout the purification of the liver enzyme as revealed by starch electrophoresis is shown in Figure 2. Slot A shows the pattern of heteromorphs in the initial crude extract that was applied to the Sepharose–Blue Dextran column. Both cytosolic (anodic) and mitochondrial (cathodic) malate dehydrogenases are resolved into multiple forms. Slot B shows that a substantial proportion of the cytoplasmic enzyme does not stick to the column under our conditions, although total retention of the mitochondrial enzyme is observed. Slot C shows that the NAD^+–L-malate wash removes the residual cytoplasmic enzyme as well as some of the less cathodic forms of the mitochondrial enzyme. Slot D shows the heteromorph pattern of the maximally purified liver m-MDH. Two to three zones of activity are observed, all of which correspond to zones of activity observed in the crude extract.

Figure 3 demonstrates the final stage in the purification of the liver enzyme. Gel A shows the multiplicity of the bands on denaturing (urea–acetic acid) electrophoresis that may be

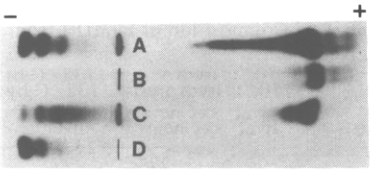

FIGURE 2: Starch gel electrophoretogram with activity staining for MDH showing isozyme patterns (top to bottom) of (A) supernatant fraction of original homogenate (0.2 U applied), (B) effluent from Sepharose–Blue Dextran column (0.02 U applied), (C) NAD^+–L-malate wash (0.07 U applied), and (D) purified m-MDH (0.06 U applied). Cathode is at the left; 0.005 M sodium citrate, pH 5.4.

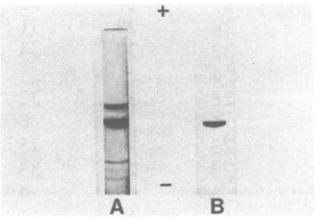

FIGURE 3: Polyacrylamide gel electrophoresis patterns of the liver enzyme before (A) and after (B) CM-52 cellulose column stepwise pH elution chromatography. Cathode is at the bottom; 6.25 M urea–0.9 M acetic acid, pH 3.2. Protein staining was with Coomassie Blue. (A) 0.035 mg of protein applied; (B) 0.03 mg of protein applied.

observed before application of the partially purified concentrated eluant from the DEAE-Sephadex column to the CM-cellulose column. Gel B shows the behavior of the pooled pH 5.55 eluate from the CM-cellulose column. Obviously, all contaminating bands of protein have been removed by the CM-cellulose treatment.

In Figure 4 the progress of the purification of the heart enzyme is illustrated, as shown by polyacrylamide gel electrophoresis. Slot 1 shows the late eluting (fractions 87–96) predominating peak of activity from the CM-cellulose column. Slot 2 shows the material from the early eluting (fractions 80–86) smaller peak of activity from the CM-cellulose column. As mentioned earlier, the early eluting peak contains more negatively charged (anodic) components by comparison with the later eluting peak. Slot 3 shows the heteromorph pattern of the crude heart homogenate before application to the blue column. In addition to the mitochondrial forms of activity, the extremely anodic cytosolic forms are also evident. Gels 1–3 were stained for enzyme activity, whereas gels 4 and 5 (purified late and early peaks, respectively) were stained for protein. It should be noted that there are no zones of protein which do not correspond to zones of activity.

Figure 5 shows the standard curve for $NaDodSO_4$–polyacrylamide slab gel electrophoresis. The mobility of each indicated standard was expressed as a fraction of the mobility of the marker dye, and this fraction is plotted against the logarithm of the molecular weight of the standard. Purified heart and liver enzyme both migrated as a single band even at high protein concentrations. Heart and liver enzymes had identical relative mobilities. A subunit molecular weight of 35 000 for both may be read from the graph.

Figure 6 shows the behavior of both heart and liver m-MDH on column isoelectric focusing using pH 9–11 ampholines. Figure 6A shows three peaks of activity for the liver enzyme at pH 9.65, 9.4, and 9.2 as well as a slight shoulder at pH 8.7–8.8. Figure 6B shows two peaks of activity for the heart enzyme at pH 9.6 and 9.4 and a shoulder at pH 8.9. While both the liver and heart enzymes appear to consist of several multiple isoelectric forms at nearly identical pH values, the distribution of these forms is clearly different in each case. Whereas the pH 9.4 peak predominated in the case of the liver

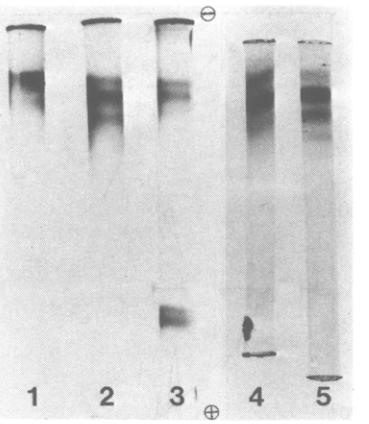

FIGURE 4: Polyacrylamide gel electrophoresis patterns of heart m-MDH both purified and in the crude homogenate. (1) Purified heart m-MDH (peak 2 from Figure 1B); (2) purified heart m-MDH (peak 1 from Figure 1B); (3) m-MDH in the initial crude heart homogenate before application to the Blue Dextran–Sepharose column; (4) purified heart m-MDH (peak 2 from Figure 1B); and (5) purified heart m-MDH (peak 1 from Figure 1B). In gels 1 and 2, 0.003 mg of protein was applied. In gels 4 and 5, 0.025 mg of protein was applied. Gels 1–3 were stained for enzyme activity. Gels 4 and 5 were stained for protein with Coomassie Blue.

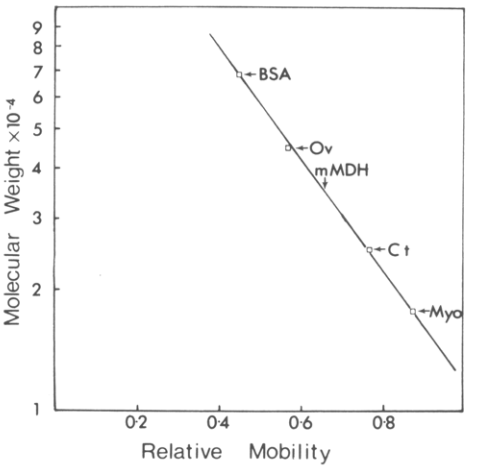

FIGURE 5: $NaDodSO_4$ electrophoresis of the purified m-MDH from heart and liver. Electrophoresis was performed in 1-mm thick slab gels as originally described by Laemmli (1970) using 12% acrylamide for the running gel and 4% acrylamide for the stacking gel. Electrophoresis was carried out for ~3 h at a constant current of 20 mA. Voltage increased from 130 to 340 V during the course of electrophoresis. Standard proteins as well as samples of purified m-MDH from liver and heart were run in different slots on the same gel. Proteins were stained with Coomassie Blue. The relative mobility of the standard proteins with respect to the tracking dye (bromophenol blue) was plotted against the logarithm of the molecular weight of these standards to give a straight line. Liver and heart m-MDH had identical relative mobilities. Only one zone of protein could be seen with both preparations even at high protein concentrations. Standard proteins were applied at 0.015 mg each while 0.010–0.040 mg of m-MDH from both liver and heart was applied. The positions of the standard proteins are indicated by horizontal arrows while the position of heart and liver m-MDH is indicated by the vertical arrow. (BSA) Bovine serum albumin; (Ov) ovalbumin; (Ct) chymotrypsinogen; and (Myo) myoglobin.

enzyme, the predominant component of the heart enzyme is the pH 9.6 peak.

Though not indicated in the diagrams for the sake of clarity, the A_{280} profile from the isoelectric column was quite consonant with the activity profile. Although the A_{280} remained slightly above zero throughout the gradient (due to the absorbance of the ampholines), there were no discernible peaks of protein which did not correspond to peaks of activity.

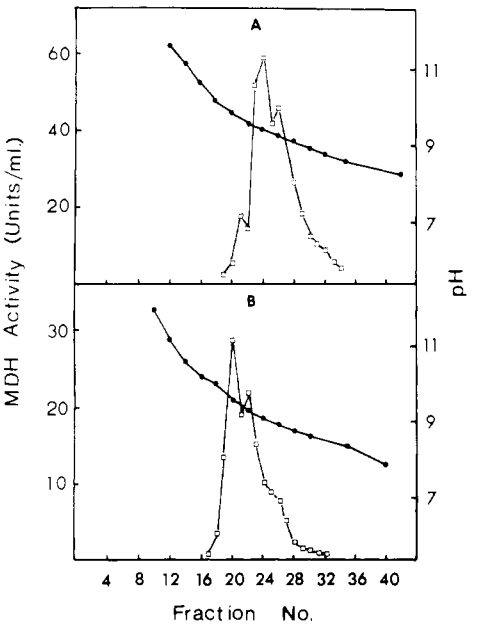

FIGURE 6: Column isoelectric focusing of purified m-MDH from rat liver and heart in a sorbitol-stabilized narrow range (pH 9–11) gradient. Electrofocusing was allowed to proceed for 50 h at 4 °C and a constant voltage of 900 V (voltage was gradually increased to this value during the first 3 h of focusing). (A) Purified liver m-MDH (4.0 mg of protein applied) and (B) purified heart m-MDH (2.0 mg of protein applied) [(□) m-MDH activity; (●) pH].

The enzyme purified from both liver and heart appeared homogeneous when subjected to denaturing and nondenaturing polyacrylamide electrophoresis as well as column isoelectric focusing. In view of these indications of homogeneity as well as those derived previously in this laboratory by sedimentation velocity analysis and slab gel isoelectric focusing, we considered the enzymes from both sources suitable for amino acid analysis. Accordingly, Table II shows the results of amino acid analysis for enzyme from both sources. Three hydrolysis times were employed (22, 46, and 72 h) at each of two hydrolysis temperatures (110 and 138 °C). Results are expressed as residues per subunit of 35 000 molecular weight for enzyme from both sources as derived from the $NaDodSO_4$ electrophoresis (Figure 5). The isoleucine peak is partly converted to D-alloisoleucine during the 138 °C hydrolysis so that values for isoleucine for this temperature are the sum of both peaks for isoleucine and D-alloisoleucine. There is little apparent difference in composition between the liver and the heart enzyme.

Of particular interest are the values for amide nitrogen in Table II. These values (26 for each enzyme) show that one-half of the Asp + Glu side chains in these preparations existed as Asn + Gln. When one considers the values for Lys, Arg, and His (27, 8, and 5) and calculates the pI with the aid of reasonable pK_a values (N-terminal ammonium, 9.0; Glu and Asp carboxyls, 4.0; His, 6.0; Lys, 10.5; Arg, 12.5; and C-terminal carboxyl, 2.0), the computed pI is ~10.1. Such a value for the isoelectric point will explain the cathodic character of m-MDH during starch electrophoresis at pH 5.4 or polyacrylamide electrophoresis at pH 8.3. It is certainly possible that partial deamidation could contribute to the multiplicity of cathodic species, as suggested by Glatthaar et al. (1974).

Figure 7 shows the UV spectra for enzyme purified from heart and liver. There is a very sharp peak very close to 280 nm and two smaller peaks at 267 and 261 nm. The spectra of enzyme from liver and heart are obviously very similar, although the liver enzyme has a smaller A_{256}/A_{280} ratio than either peak 1 or peak 2 enzyme from the heart.

Table II: Amino Acid Composition of m-MDH from Rat Heart and Liver[a]

residue	110 °C hydrolysis		138 °C hydrolysis	
	liver	heart	liver	heart
Lys	27	27	27	26
His[b]	5	5	5	5
ammonia[c]	26	27	28	29
Arg	8	8	8	8
Asp	26	26	26	24
Thr[d]	22	22	24	28
Ser[d]	14	14	11	12
Glu	26	25	26	27
Pro	21	20	22	25
Gly[b]	28	29	31	29
Ala[d]	32	32	34	33
Cys[e,f]	4			
Val[b]	28	30	32	30
Met	6	6	6	8
Ile[b,g]	23	21	25	24
Leu	29	28	33	31
Tyr	5	5	4	4
Phe	12	11	11	11
Trp[f]	0			

[a] Values are the average for 22-, 46-, and 72-h hydrolysis times. Hydrolysis was accomplished in 6 M hydrochloric acid in vials sealed under vacuum (0.6 mmHg). Results are expressed as residues per subunit, i.e., residues/35 000 daltons. [b] 72-h values taken. [c] Values for 22-h hydrolysis for 110 °C; extrapolation to zero time for 138 °C. [d] Values obtained by extrapolation to zero time. [e] Quantitated as cysteic acid after performic acid oxidation. [f] Determined separately. [g] Includes peak values for D-alloisoleucine in 138 °C hydrolysis.

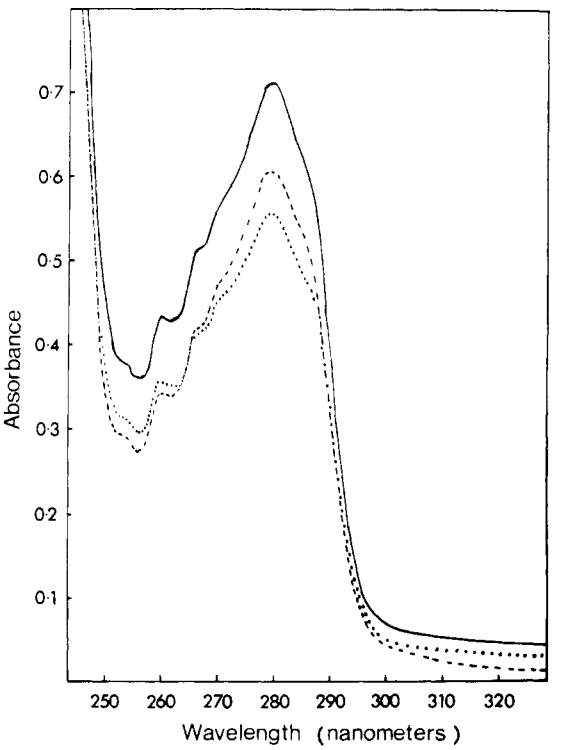

FIGURE 7: UV spectra of purified mitochondrial malate dehydrogenase from rat liver and heart. (—) Rat heart m-MDH (peak 2 from Figure 1B, 310 U/mL); (---) rat liver m-MDH (220 U/mL); and (···) rat heart m-MDH (peak 1 from Figure 1B, 210 U/mL). The spectra represent the absorbance of the above m-MDH solutions against 20 mM citrate, pH 5.55, as a blank.

Discussion

Recently, the value of Sepharose–Blue Dextran or Sepharose–Cibacron Blue in purification of nucleotide-requiring

enzymes has become apparent (Thompson et al., 1975; Wilson, 1976). Though some kinases have been purified by using Blue Dextran interactions (Diezel, et al., 1973), the immobilized dye is proving of most value in the purification of NAD- or NADP-dependent dehydrogenases. Ryan & Vestling (1974) described a successful purification procedure for lactate dehydrogenase from rat liver using Sepharose–Blue Dextran pseudoaffinity chromatography. This purification was both mild and rapid, yielding apparently homogeneous enzyme of high specific activity in under 1 day. In view of the great advantage of this technique in shortening established purification procedures and improving yields, the potential of using Sepharose–Blue Dextran for purifying mitochondrial malate dehydrogenase was examined.

At low ionic buffer concentration, m-MDH and, to a much smaller extent, cytoplasmic malate dehydrogenase were found to bind to Sepharose–Blue Dextran resin as shown in Figure 2. Essentially all of the mitochondrial enzyme is bound to the Sepharose–Blue Dextran resin if the column is not overloaded. (By use of a 24 × 5 cm column, up to 300 g of tissue may be processed without overloading.) Most of the cytoplasmic MDH is found in the effluent. m-MDH can be removed by eluting with 0.1–0.2 M KCl, resulting in several-fold purification. However, subsequent separation of contaminating proteins such as lactate dehydrogenase is far from satisfactory. Rhodes et al. (1964) succeeded in eluting MDH from *Ascaris suum* from a DEAE-cellulose column by using NAD^+–L-malate. Yoshida (1970) reported the purification of glucose-6-phosphate dehydrogenase and other enzymes by selective elution with substrate analogues from an ion exchange column. In the present study with the concentration of NAD^+–L-malate used, cytoplasmic and small amounts of mitochondrial MDH were eluted from the column (Figure 2).

In order to strip lactate dehydrogenase (LDH) from the column, we used NADH followed by NADH–oxamate. These steps are necessary because subsequent purification of m-MDH by DEAE-Sephadex A-50 and CM-52 cellulose ion-exchange chromatography failed to remove LDH completely. Gel filtration on Sephadex G-100 and G-200 resulted in only partial separation of the two enzymes. It should be stressed that by using the purification procedure described in this communication in conjunction with that described previously for LDH (Ryan & Vestling, 1974), both m-MDH and LDH may be purified simultaneously in good yield.

Recently, O'Carra et al. (1974) reported affinity chromatographic separation of LDH isoenzymes on the basis of differential abortive complex formation. In a preliminary report (Kuan & Vestling, 1975) we had shown that m-MDH may be eluted from the Sepharose–Blue Dextran column in an abortive ternary complex with NAD^+ and oxaloacetate. However, subsequent steps proved less than satisfactory. As a consequence, NADH and sodium D(+)-malate were used to form an abortive ternary complex as reported by Theorell & Langan (1960).

The purity of the malate dehydrogenase is as high in terms of specific activity as that prepared previously in this laboratory by a much more laborious procedure (Sophianopoulos & Vestling, 1962). It is rather difficult to compare specific activity directly with reports from other laboratories since assay procedures vary widely. However, it is quite evident that in terms of yield, overall purification, and criteria of homogeneity our procedure for purification of rat heart and liver mitochondrial MDH represents an improvement in comparison to procedures recently described for rabbit heart m-MDH (Dölken et al., 1974) and pig heart m-MDH (Glatthaar et al., 1974). In addition, the mildness of our extraction procedure is much more likely to preserve the protein in its native state. Furthermore, the rapidity with which the purification may be performed (about 48 h) and the inclusion of protease inhibitors would appear to obviate the problems of artifactual generation of multiple forms by partial proteolysis (Cassman & King, 1972) and/or partial deamidation (Noyes et al., 1974; Glatthaar et al., 1974) during the course of enzyme purification.

Very recently Hägele et al. (1978) reported a successful purification for both cytoplasmic and mitochondrial forms of malate dehydrogenase from acetate-grown yeast using Sepharose–Blue Dextran (Ryan & Vestling, 1974) and Blue Dextran–polyacrylamide gel chromatography as an initial step. Cytoplasmic enzyme was eluted with 2 mM NADH in 20 mM Tris-HCl, pH 7.0, and mitochondrial enzyme was eluted by 0.5 M KCl in the same buffer. While Sepharose–Blue Dextran absorption appeared to be an excellent first stage in the purification of the yeast enzymes reported by these workers, elution procedures and subsequent purification steps are clearly not completely satisfactory since overall recoveries are very low. Walk & Hock (1976) have reported the separation and subsequent purification of cytoplasmic, mitochondrial, and glyoxysomal malate dehydrogenases from watermelon cotyledons using 5′-AMP–Sepharose as an affinity adsorbent. The parallel use of Sepharose–Blue Dextran and 5′-AMP–Sepharose in the purification of the same enzyme, albeit from different sources, is a practical vindication of the proposal of Thompson et al. (1975) that the blue dye acts as an adenine nucleotide analogue.

The presence of multiple electrophoretic forms of m-MDH has been variously attributed to partial proteolysis and/or partial deamidation as explained previously, to conformational changes (Kitto et al., 1966), and to differences in the amounts of covalently bound phosphate (Cassman & Vetterlein, 1974).

Mann & Vestling (1968, 1969, 1970) demonstrated the somewhat variable presence during starch gel electrophoresis at pH 5.4 of two or three cathodic forms of m-MDH. They proposed an explanation based on the existence of dissimilar subunits which were shown to be capable of dissociating and reassociating under such conditions as brief exposure to low pH. This explanation was a reasonable basis for viewing the results obtained with enzyme from the older multistep procedure (Sophianopoulos & Vestling, 1962). However, it is clear that our new procedure (which, by virtue of its rapidity and inclusion of protease inhibitor, reduces the possibility of enzymic degradation) leads to a single subunit species in good yield for both heart and liver enzyme. This result is obtained in denaturing gels of the urea–acetic acid or $NaDodSO_4$ type, and so we are led to the conclusion that the original observations of dissimilar subunits for m-MDH from rat liver (Mann & Vestling, 1970) resulted from artifactual changes introduced during the purification procedure. Indeed, if the protease inhibitor is omitted and the original extract allowed to stand for 40 h before application to the Sepharose–Blue Dextran column, a faint extra band may be observed upon urea–acetic acid denaturing polyacrylamide electrophoresis. We suggest that the earlier explanation of dissimilar subunits be abandoned and that m-MDH of rat liver and heart be considered a homodimer.

The results presented in Figures 2 and 4 show that the m-MDH isolated by our new procedure from liver or heart nevertheless *does* consist of several multiple forms. It is clear that these multiple forms correspond precisely to forms observed in fresh homogenates of either liver or heart. Ap-

parently, the existence of these multiple forms may not be rationalized as artifacts of the purification procedure (Glatthaar et al., 1974). In Figure 6A we showed that we may separate m-MDH from liver into four isoelectric forms at pH 9.65, 9.4, 9.2, and 8.75. In Figure 6B we showed that the heart enzyme had a different distribution of activity, but isoelectric forms at nearly identical pH values (9.6, 9.4, and 8.9) may be resolved. Dölken et al. (1974) have recently resolved purified rabbit heart m-MDH into three isoelectric forms at pH 9.5, 9.2, and 9.0.

In attempting to probe the meaning of these multiple isoelectric forms of m-MDH and specifically to try to rationalize the difference in the distribution of these forms in the liver and the heart, we subjected enzyme from both sources to amino acid analysis. These results are expressed in Table II. It is quite evident that there is no appreciable difference in composition between the two sources of the enzyme. The compositions of heart and liver enzyme differ only slightly from the compositions of other previously reported mammalian heart mitochondrial malate dehydrogenases (Banaszak & Bradshaw, 1975). As would be expected on simple evolutionary grounds, much more marked differences exist between the composition of our m-MDH from rat liver and heart and the compositions of chicken heart, tuna heart, and yeast m-MDHs (Banaszak & Bradshaw, 1975; Hägele et al., 1978). It is also apparent that different mammalian mitochondrial malate dehydrogenases are far more similar in composition, one to the other, than are the mitochondrial and cytoplasmic malate dehydrogenases from any one given mammalian species.

Significant differences did seem to occur between our 110 and 138 °C hydrolyses for enzyme, from either source. Values for the aliphatic amino acids tended to be slightly higher at the higher temperature. This may reflect incomplete hydrolysis at 110 °C (Moore & Stein, 1963). On the other hand, the value for serine is considerably lower at the higher temperature. This amino acid is, of course, very labile. Values for ammonia released from asparagine and glutamine were consistently slightly higher in the case of the heart enzyme than for the liver enzyme. This could possibly explain the difference in isoelectric heteromorph distribution between the two sources of m-MDH since it allows calculation of a slightly higher p*I* for the heart enzyme in agreement with the results of Figure 6.

Very recently, however, we investigated other possible sources of the isoelectric heterogeneity of m-MDH from heart and liver (Jones & Vestling, 1978a). We have shown that subjecting enzyme from either source to a freeze–thaw cycle with lysolecithin abolished the multiplicity in both cases and converted both liver and heart enzyme to a single isoelectric form at pH 9.6. We have subsequently established, in agreement with Dodd (1973), that addition of lysolecithin stabilized the enzyme toward heart inactivation.

When malate dehydrogenase from crude fresh rat heart extracts is separated by slab gel isoelectric focusing (Jones & Vestling, 1978a), we observe a spectrum of m-MDH forms spanning the gap between the m-MDH isoelectric forms at pH 9.6–8.9 and the supernatant MDH isoelectric forms at pH 5.2–5.6. Furthermore, we may generate acidic, lower p*I*, supernatant-like forms of malate dehydrogenase by treatment of crude extracts or purified m-MDH with phospholipase D. We have been able to demonstrate the presence of variable but significant amounts of phospholipid and neutral lipid in our purified enzyme from either heart or liver (Jones & Vestling, 1978b). It is possible that masking of ionic side-chain groups by tightly bound phospholipid, as proposed by Rosseneu et al. (1977) and Soetewey et al. (1977) for human plasma high-density lipoproteins, may contribute to the existence of multiple cathodic electrophoretic species of m-MDH. The relatively high level of amidation of Asp and Glu side chains which obtains when our rapid, "gentle" isolation procedure is employed is largely responsible for the high isoelectric points observed. To the extent that the presence of bound phospholipid in "pure" m-MDH reflects the condition of the enzyme in the intact heart or liver cell and does not reflect the failure to remove traces of adventitiously bound lipid acquired during isolation, one can postulate that complexation with phospholipid may serve to protect the amide status of m-MDH. This may be important for the functioning of the enzyme in the cell, and we are studying this possibility.

There is evidence that m-MDH has structural properties which can result in its reversible association with the inner mitochondrial membrane in response to changing metabolic states (Waksman & Rendon, 1971). We have reason to believe that the heterogeneity of native m-MDH from rat heart and liver is caused by differential lipid binding. The observed multiple forms of m-MDH then may reflect a dynamic equilibrium between malate dehydrogenase that is free in the intermembranal matrix and that which is bound to the inner membrane.

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Interaction of the Substrate Phosphate Substituent with Glyceraldehyde-3-phosphate Dehydrogenase†

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ABSTRACT: Linear free energy relationship studies were carried out to examine the nature of the interaction of aldehydes with glyceraldehyde-3-phosphate dehydrogenase. The association constant of most aldehydes with the enzyme is dependent on the Taft polar substituent constant, characterized by a ρ^* value of 1.7. This is identical with the ρ^* value for thiohemiacetal formation between aldehydes and glutathione and suggests that the binding of aldehydes to the enzyme results in the formation of a thiohemiacetal adduct. D-Glyceraldehyde 3-phosphate, however, shows a positive deviation from the linear correlation, indicating a specific interaction of the phosphate substituent with the enzyme. Thiohemiacetal formation contributes 3.9 kcal/mol to the formation of the enzyme–aldehyde complex with D-glyceraldehyde 3-phosphate, and the interaction of the phosphate moiety on the aldehyde with the phosphate binding site on the enzyme contributes 3.7 kcal/mol. The interaction of inorganic phosphate with the aldehyde phosphate binding site on the enzyme is characterized by a $\Delta G^{\circ\prime} > -1.4$ kcal/mol, indicating that most of the apparent free energy for binding the phosphate substituent on the aldehyde is entropic in origin. Phosphorylation of irreversible inhibitors of glyceraldehyde-3-phosphate dehydrogenase has been shown to enhance their reactivity with the enzyme by 3.8 (±1) kcal/mol [Byers, L. D. (1977) *J. Am. Chem. Soc. 99*, 4146–4149]. Phosphorylation of the aldehyde substrates also enhances the stereoselectivity of the overall oxidative reaction [Byers, L. D. (1978) *Arch. Biochem. Biophys. 186*, 335–342]. In addition to these effects, and the enhanced binding of aldehydes, the phosphate substituent was found to alter the conformational mobility of the protein, the specificity of deacylation of the acyl enzyme (with respect to acyl acceptors), and the reactivity of the aldehydes as substrates in the overall oxidative arsenolysis reaction. On the basis of electronic effects of the acyl substituent, D-glyceraldehyde 3-phosphate is more reactive than are nonphosphorylated aldehydes by a factor of 10^4.

Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) catalyzes the oxidative phosphorylation of D-glyceraldehyde 3-phosphate. The early studies of Racker & Krimsky (1952) and of Harting & Velick (1954) have demonstrated that glyceraldehyde-3-phosphate dehydrogenase (from yeast and rabbit muscle) can also catalyze the oxidation of a variety of aldehydes but at substantially reduced rates relative to that for oxidation of D-glyceraldehyde 3-phosphate. In addition to enhancing the reactivity of the aldehyde, the phosphate substituent enhances the stereoselectivity of the enzyme. D-Glyceraldehyde 3-phosphate is at least 30 000 times more reactive than L-glyceraldehyde 3-phosphate, but D-glyceraldehyde is only 180 times more reactive than its enantiomer (Byers, 1978). The reaction of irreversible inhibitors with the enzyme is also enhanced by the presence of a phosphate moiety. After correction for electronic effects (Byers, 1977), glycidol phosphate is found to be 630 times more reactive than other epoxides with the enzyme, and chloracetol phosphate is found to be 680 times more reactive than other modifying reagents with the enzyme (Byers, 1977). This enhanced reactivity of the phosphorylated irreversible inhibitors with the enzyme (which corresponds to a lowering of the free energy of activation by 3.8 ± 0.1 kcal/mol) is a measure of the specific interaction of the phosphate moiety with the enzyme. This study was undertaken to examine the nature of the interaction of the phosphate substituent with the enzyme and evaluate the role of this interaction in the binding and catalytic steps of the enzyme reaction.

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