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HETEROGENEITY OF SUPERNATANT MALATE DEHYDROGENASE

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SUMMARY

A way was found to demonstrate electrophoretically that the supernatant portion of pig heart malate dehydrogenase (L-malate:NAD oxidoreductase, EC 1.1.1.37) is heterogeneous. Other pig organs displayed the same pattern of supernatant malate dehydrogenase forms. Heterogeneity of the supernatant malate dehydrogenase from tissues of various laboratory animals was also observed, the patterns showing species differences in each case. The pig heart supernatant fractions after electrophoresis were not distinguishable from each other by a number of degradative treatments nor by their reaction with several coenzyme analogs. One major form is selectively inactivated by precipitation with ammonium sulfate; also it may be formed through urea or acid inactivation of the other remaining electrophoretic units followed by reactivation steps. Possible structural relationships between the supernatant electrophoretic fractions are discussed.

INTRODUCTION

The existence of multiple forms of many enzymes has been widely reported and reviewed[1-3]. In the case of malate dehydrogenase (L-malate:NAD oxidoreductase, EC 1.1.1.37), two forms are regularly encountered in a single tissue, one often claimed to be of mitochondrial origin, the other easily extractable and referred to as the supernatant or cytoplasmic form. That this may be misleading is shown by the demonstration[4] of 15% malate dehydrogenase activity in rat liver mitochondria as "supernatant" in character and roughly 10% of the activity in the supernatant fraction as "mitochondrial" in type. The microsomes have been found to contain 50% of each kind. In the case of beef heart[5-7] and also of pig heart[8,9] the two kinds above differ from one another in catalytic, chromatographic, and electrophoretic properties as well as in amino acid composition. Recently mitochondrial malate dehydrogenase was reported to have been resolved into five active fractions by starch gel electrophoresis[10]. In the present paper the resolution of pig heart supernatant-derived malate dehydrogenase into a number of active forms is described and the nature of the heterogeneity is discussed.

MATERIALS AND METHODS

Pig hearts, cooled immediately in ice after sacrifice, were trimmed, rinsed with cold water, either frozen immediately or homogenized and used within 1 h. If frozen, they were stored at -15° for 4 or 5 days only. An acetone powder from a single adult pig heart was stored at -15°. The rat, guinea pig, and pigeon organs were obtained with ether anesthesia or decapitation. Co-enzyme analogs were purchased from P–L Biochemicals, Inc., Milwaukee, Wisc. Connaught Labs., Toronto, Canada, supplied the hydrolyzed starch; trypsin ("1:200") was obtained from Difco Labs., Detroit, Mich.

Electrophoresis was performed in a manner similar to that described by SMITHIES[11]. Short filter paper wicks connected the buffer compartments to the gels. The gel trays measured 23.5 cm × 2.0 cm × 0.6 cm. Buffers were prepared by diluting a stock solution (0.20 M maleic acid, and 0.20 M Tris) with water and NaOH to the desired concentration and pH. Gels composed of 14% (w/w) starch were used throughout. The gels were made up with a Tris–maleate buffer (0.015 M) at pH 7.0 and the reservoirs were filled with the same at 0.02 M (with respect to maleate).

Tissues were prepared for electrophoresis by grinding them with 0.02 M potassium phosphate buffer (pH 7.0) in a glass homogenizer and clarifying by centrifugation. The malate-dehydrogenase activity of preparations used for electrophoresis varied up to 130 enzyme units/ml. The materials were added to the gel on pieces of Whatman 3 MM filter paper; the gels were then covered with paraffin oil and subjected to electrophoresis for 20–24 h at 4°, employing a voltage drop of 7 to 8 V/cm measured near the wick-gel junctions.

Malate dehydrogenase activity was determined by the tetrazolium and spectrophotometric methods of THORNE, GROSSMAN AND KAPLAN[10], rates by spectrophotometer being calculated in terms of μmoles of coenzyme reduced per min. Tetrazolium staining was carried out for constant time periods which were appropriately chosen to show relative rates. Protein concentration was estimated by assuming that 1.0 mg of protein per ml has an absorbance of 1.0 at 280 mμ.

RESULTS

Electrophoretic separation

Fig. 1 shows the malate dehydrogenase pattern of a pig heart, acetone powder homogenate subjected to electrophoresis under standard conditions (pH 7.0). The mitochondrial forms (low cathodic mobility)[10] are only partially resolved under these conditions but the 3 major forms of supernatant malate dehydrogenase are clearly visible migrating toward the anode. The gel was lightly stained to obtain maximum resolution. In Fig. 2 several weaker bands that become visible upon further staining,

Fig. 1. Malate dehydrogenase pattern of a homogenate of a single adult pig heart acetone powder. Starch gel electrophoresis performed using maleate–Tris buffer at pH 7.0 and light staining as described in text.

are shown schematically. Qualitatively, forms 4, 5, and 6 (more appropriately, D, E, and F), appear to comprise about 90% of the total supernatant malate-dehydrogenase activity, the distribution among the 3 being in the approximate ratio 1:1:2, respectively. An identical pattern was observed in 5 fresh individual adult hearts and the pattern was not altered by freezing or freeze-drying the tissues. Storage at −15° for more than a year did not change the electrophoretic patterns.

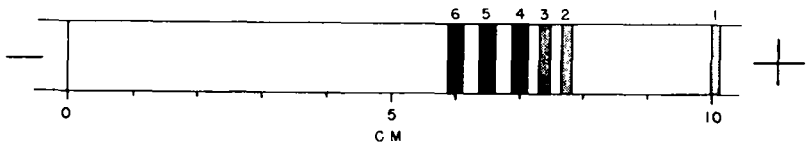

Fig. 2. A schematic representation of the total supernatant malate dehydrogenase pattern of pig heart homogenate obtained by starch gel electrophoresis using maleate Tris buffer (pH 7.0) and prolonged staining as described in the text. Bands A–F are here 1–6.

Tris–acetate and Tris–malate buffer solutions at pH 7.0 produced the same pattern as did the Tris–maleate. On the other hand citrate–phosphate (pH 7 or 8.7), sodium borate (pH 8.5), Tris–borate (pH 8.5) and Tris–citrate (pH 7.0) did not resolve the 3 major fractions of supernatant malate dehydrogenase and the enzyme migrated as a broad smear of activity. In some of these cases, band 1 (more appropriately, A), Fig. 2, was observed even though the other bands were not resolved. Tris–acetate was not effective at pH 8.5, while Tris–maleate was effective at 7.6 and gave only partial resolution at 6.0.

When bands 4, 5, and 6 were excised with pieces of gel and placed at the origin of 3 fresh gels they migrated as single bands back to a position similar to that occupied in the original gel. Also, in the course of some 20 standard runs the distribution patterns were invariably the same except that band I was sometimes not detectable. The homogenate from an acetone powder could be stored at 4° and subjected to electrophoresis several days in succession without any change of distribution pattern or loss of malate dehydrogenase activity. In 2 cases, band 1 appeared only after storage at 4° for 1 or 2 days. Occasionally band 1 did not become visible in homogenates aged as long as 3 days.

Pattern from other sources

Using the same techniques, an essentially identical pattern was found in preparations of pig liver, kidney and diaphragm. The heart and liver supernatant malate dehydrogenase electrophoretic patterns of the rat (Sprague-Dawley) and the guinea pig each showed a single major band, the rat having a mobility corresponding to pig heart malate dehydrogenase band 6 and the guinea pig having a mobility 65% lower. Within either one of these species the heart and liver showed almost identical patterns. Several other bands of supernatant malate dehydrogenase were seen but these constituted only a very small fraction of the total activity. From pigeon heart the supernatant malate dehydrogenase was localized in a single band with about 55% of the mobility of pig heart malate dehydrogenase band 6. Two weak bands were observed close by on either side of the major band and a very weak additional band was noted with the same mobility as malate dehydrogenase band 6.

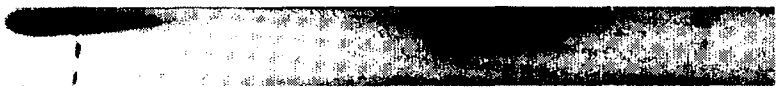

Fig. 3. Effect of trypsin on pig heart malate dehydrogenase pattern. Starch gel electrophoresis and staining as described in text. Top, no trypsin; middle, treated with 2 mg/ml trypsin for 1 h at 37° (pH 10); lowest, treated for 2 h with trypsin.

Characteristics of the bands

To test the effect of trypsin, samples of pig heart homogenate were diluted 1:1 in 0.01 M sodium glycinate buffer (pH 10.0) containing 2 mg/ml of trypsin. Fig. 3 shows the malate-dehydrogenase pattern of pig heart homogenate untreated, treated with trypsin for 1 h at 37°, and treated for 2 h prior to electrophoresis. In 1 h the mitochondrial malate-dehydrogenase activity is no longer detectable but supernatant bands 4, 5, 6, and 1 are still visible, the distribution of activity remaining apparently unchanged. After 2 h of trypsin digestion no malate-dehydrogenase activity can be seen.

Fig. 4. Effect of heat on pig heart malate dehydrogenase pattern. Electrophoresis and staining as described in text. Top, blank homogenate; middle, heated at 55° for 1 min; lowest, heated at 55° for 2 min.

Thermal stability is shown in Fig. 4, the mitochondrial malate dehydrogenase being almost completely inactivated after 1 min at 55°, one weak band being relatively unharmed (corresponding to D or E, THORNE, GROSSMAN AND KAPLAN[10]). After 2 min there is practically no mitochondrial malate- dehydrogenase activity left but supernatant activity is unharmed. By increasing the time or temperature it becomes apparent that the supernatant forms are inactivated at similar rates as evidenced by a uniform fading of the pattern. After 2 min at 66° no malate-dehydrogenase activity is detectable.

Exposure of pig heart homogenate to 3.0 M urea for 15 min at 37° led to disappearance of all mitochondrial malate dehydrogenase (save a faint band, THORNE's D or E) but the supernatant pattern was not affected by this treatment. 3.5 M urea destroyed all mitochondrial activity and reduced the supernatant forms greatly and uniformly. At 4.0 M urea there was total loss of activity. Likewise the mitochondrial forms appear to be more sensitive to both acidic and basic solutions.

To test the effects of coenzyme analogs, 4 gel strips, each containing 3 bands, 4, 5, and 6, were incubated in the dark for 1 h at 37° in 4 different nitro-blue tetrazolium solutions, having as coenzyme content respectively: NAD^+, nicotinamide hypoxanthine, 3-acetyl pyridine, and thionicotinamide coenzyme analog, each at 0.3 mg/ml. The strip developed with NAD^+ appeared somewhat darker than the others. A check of its progress during the incubation period showed that the color of the NAD^+ strip developed sooner than the others. Assuming that the reduced coenzymes reduced nitro-blue tetrazolium at equal rates, it would appear that of the 4, NAD^+ is the coenzyme most easily utilized by supernatant malate dehydrogenase. This qualitative

TABLE I

RELATIVE REACTION RATES OF PARTIALLY PURIFIED PIG HEART SUPERNATANT MALATE DEHYDROGENASE WITH COENZYME ANALOGS

Results reported as the ratios of the rates of reaction of the enzyme preparation with NAD as proton acceptor to the reaction rates with coenzyme analogs as acceptors (NHD, nicotinamide hypoxanthine dinucleotide; TNAD, thionicotinamide adenine dinucleotide; APAD, 3-acetylpyridine adenine dinucleotide.)

$\frac{NAD}{NHD}$	2.8	2.2*
$\frac{NAD}{TNAD}$	2.1	2.1*
$\frac{NAD}{APAD}$	2.5	2.3*

* Rate ratios reported by THORNE, GROSSMAN AND KAPLAN[10] obtained under the same conditions with a pig heart supernatant malate-dehydrogenase preparation.

observation was confirmed by determining reaction rates spectrophotometrically[10] with partially purified supernatant malate dehydrogenase (see below), the results being reported as the ratio of the rate obtained with NAD^+ to the rate obtained with analog in Table I. The analogs at 0.3 mg/ml reacted at a rate $^1/_2$ to $^1/_3$ that of NAD^+. The 3 major forms of pig heart supernatant malate dehydrogenase were not differentiated by individual analogs, all of which gave a 1:1:2 pattern of color intensity.

To test the possibility that the bands occur as a consequence of complexing of enzyme and small molecule, one pig heart homogenate was dialyzed 48 h against a large volume of buffer. Another homogenate was precipitated with acetone at 0° and stirred for 10 min; the precipitate was collected by centrifugation and returned to its original volume with buffer. A third sample was treated with Norit charcoal (10 mg/ml), allowed to stand 15 min and centrifuged to remove the charcoal. The Norit treatment was repeated again. None of the 3 showed a change in malate-dehydrogenase activity or band pattern or a visible shift in intensity of color.

Partially purified supernatant malate dehydrogenase

Since extraction of the individual bands from the gel after electrophoresis resulted in very low yields (never more than 10% of the activity), fractionation of 200 g of mince from a single adult pig heart was attempted by the methods of ENGLARD AND BREIGER[12] and THORNE[13]. Steps 1, 2, and 3 (extraction with sucrose solution, $(NH_4)_2SO_4$ precipitation, and heat-treatment) were used without modification. The enzyme preparation obtained from step 3 was subjected to chromatography with DEAE-cellulose, eluting first with 0.01 M potassium phosphate at pH 6.9 to eliminate the mitochondrial enzyme, and then with a linear concentration gradient (0.01 to 0.05 phosphate) to recover 39% of the total activity applied.

This material retained its high activity when subjected to freeze-drying and storing at −15° for more than 4 months. Electrophoresis of early and late fractions from the column showed no change in pattern of distribution, as shown in Fig. 5 (above), where bands 2 to 6 (more appropriately, B–F), are visible, but neither mitochondrial bands nor band 1 are seen. Identities were confirmed by subjecting crude

Fig. 5. Above, electrophoretic pattern of partially purified supernatant pig heart malate dehydrogenase; below, electrophoretic pattern of partially purified malate dehydrogenase subjected to urea deactivation followed by reactivation. Starch gel electrophoresis and staining as described in text.

homogenate to electrophoresis in the same gel. The most remarkable difference between the partially purified supernatant malate dehydrogenase and that of the crude homogenate was a great diminution in the activity of band 6. From being roughly twice as enzymatically active as bands 4 or 5, it became considerably less active in the course of purification. Bands 4 and 5 maintained a 1:1 activity ratio.

By monitoring each step of the purification it was found that band F was diminished in activity after the first $(NH_4)_2SO_4$ treatment, the resulting pattern being maintained throughout the rest of the purification. In another experiment a 0.25 M sucrose extract of pig heart mince was brought successively to 20, 30, 40, 50, 60, 70, 80, and 100% of saturation by the addition of $(NH_4)_2SO_4$ and the precipitates at each step collected by centrifugation. Most of the activity was found in the precipitate from 50–60, 60–70, 70–80, and 80–100% saturated $(NH_4)_2SO_4$ fractions, which contained 0.72, 3.9, 22.0, and 12.0% of the starting malate-dehydrogenase activity. Band 1 and the mitochondrial enzyme were not seen in the 80–100% fraction but appeared in peak quantities in the 60–70% fraction. Bands 2 through 5 were present in all the fractions with no variation in relative intensity. All the patterns were similar to that in Fig. 5 (above), band 6 being greatly reduced in relative intensity. The latter seemed to have been largely destroyed by the ammonium sulphate treatment. Bands 2 (more appropriately, B), and 3 appear to gain in sharpness and intensity concurrent with the disappearance of band F.

Inactivation and reactivation

Malate dehydrogenase of vertebrate origin has been shown to be composed of two or more noncovalently bound subunits which may be reversibly dissociated by appropriate treatment. Dissociation and hybrid formation has been achieved with mitochondrial and supernatant malate dehydrogenase[14,15]. A pig-heart acetone homogenate was diluted 1:40 with cold solution of 8.0 M urea in 0.1 M 2-mercaptoethanol. After 15 min at 0°, no malate dehydrogenase activity was detectable; but 58% of the original was recovered after dialyzing against 50 vol. of a solution of 0.1 M sodium citrate and 0.1 M 2-mercaptoethanol (pH 7.0) for 24 h at 4°. After concentration by dialysis against polyethylene glycol there was no loss of activity and electrophoresis showed no change in malate dehydrogenase pattern except a considerable weakening of the mitochondrial smear. No hybrid malate dehydrogenase of intermediate mobility was noted.

It is of interest that by the same urea treatment the partially purified, supernatant malate dehydrogenase, deficient in band 6, was reconstituted by sodium citrate dialysis to the familiar 1:1:2 ratio of the crude preparations, as shown in Fig. 5. The high degree of reactivation (75%) precludes partial destruction of the activity of bands 4 and 5 as an explanation of the shift. It appears rather that bands 4 and 5 were con-

verted to band 6 in the process of reactivation and that each contributed equally, since the two remained in the ratio of 1:1. In an attempt to see if a similar redistribution would take place under conditions known to dissociate lactate dehydrogenase[2,3], a sample of partially purified malate dehydrogenase in 0.01 M potassium phosphate (pH 7.0) was diluted 1:1 with 2 M NaCl and frozen. After 18 h at −15°, there was no change in enzyme activity nor, after electrophoresis, any in band pattern.

Also partially purified supernatant malate dehydrogenase in 0.1 M phosphate buffer was diluted with 0.3 M 2-mercaptoethanol to give a solution 0.1 M in mercaptan and held at pH 2 (HCl) for 5 min at 25°, whereupon no malate dehydrogenase activity could be detected. Sodium citrate produced 30% reactivation in 1 h to original bands. The same result with only 3% recovery was found with 0.25 M sucrose extract of freeze-dried pig heart tissue.

Extraction

Finally, as noted above, an attempt was made to extract the constituents of each band from the gel in order to carry out similar tests on the isolated forms. This effort at extraction produced such a small fraction of each band that it was felt testing would be unreliable.

DISCUSSION

In contrast to the electrophoretic distribution pattern of the lactate dehydrogenase system[16,17], tissue specificity of malate dehydrogenase was not found in the pig but species specificity (pig, rat, guinea pig, pigeon) was definitive. The lack of resolution of the various forms in many buffer systems suggests the possibility of their differences being small or of some kind of interaction between the fractions. The differential deactivation of the mitochondrial forms of pig heart malate dehydrogenase by heat, trypsin, urea, acid, and base may be contrasted to the equality of these reactions for the individual supernatant bands here described; a similar contrast exists in the ratio of reaction rates with several coenzyme analogs. Large stability and catalytic differences exist between the isoenzymes of lactate dehydrogenase[18].

The heterogeneity of supernatant malate dehydrogenase may be caused simply by conformational differences, as suggested for the various forms of mitochondrial malate dehydrogenase by KITTO, WASSERMAN AND KAPLAN[24], in view of the lack of tissue specificity, the difficulty of separation of the bands in several buffers, and the equal reactions of the forms to deactivation or to presence of analogs. The results of the deactivation–reactivation steps are also capable of a similar interpretation and this is re-enforced by the absence of hybrids. On the other hand, the changes observed might be explained by the following:

It may be postulated that the components F, E, and D (6, 5, and 4, respectively, in Fig. 2) are dimeric molecular combinations of two similar but not identical polypeptide chains. Thus F could be produced by the combination of the two chains, E by a dimer composed of two molecules of one of the chains, and D by a dimer of two molecules of the other chain. Recently an NADP isocitrate dehydrogenase isoenzyme system was suggested to be of this same type[19]. The dimer composed of two different chains was reported to have an electrophoretic mobility intermediate between the other two dimers, each composed of two identical chains. Similarly, the hybrid forms

of lactate dehydrogenase have intermediate mobilities and properties[20]. There is no logical necessity for such behavior, and in the present situation the dimer F, with a lower mobility than the others, would probably be the one composed of two different chains; it also exhibits a sensitivity to ammonium sulphate not shared by the other two forms.

By this hypothesis the combination of the sub-units to form three active dimers would appear to be random both *in vivo* and *in vitro* because the 1:1:2 activity distribution of the D, E, and F bands is unchanged after the urea association-recombination step. Band A (1 in Fig. 2) has a much higher electrophoretic mobility than the others as well as a lower solubility in ammonium sulphate solutions, and so may be different structurally. It could be a non-specific dehydrogenase. Bands C, and B (3 and 2, respectively, in Fig. 2) showed some suggestive conversion from band F when the latter was obliterated by treatment with ammonium sulphate; this and their 1:1 ratio raised the possibility that they are really the two chains existing together as dimers in bands F, E, and D. It should be noted that low molecular units of mitochondrial malate dehydrogenase have been isolated[21,22] and HENDERSON has suggested heterogeneity for supernatant malate dehydrogenase[23].

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