Bovine Heart Malic Enzyme

I. ISOLATION AND PARTIAL PURIFICATION OF A CYTOPLASMIC AND A MITOCHONDRIAL ENZYME*  

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SUMMARY

Homogenates prepared from bovine heart muscle contain two distinct forms of malic enzyme (malate:NADP+ oxido-reductase (decarboxylating), EC 1.1.1.40), which can be easily separated by chromatography on DEAE-cellulose. A partial purification of the two enzymes has been accomplished by means of DEAE-cellulose chromatography, ammonium sulfate fractionation, and chromatography on CM-Sephadex. The isolated fractions migrate at different rates on disc gel electrophoresis and appear to have a similar molecular weight (approximately 200,000) as determined by sucrose density gradient centrifugation and gel filtration.

Malic enzyme has also been purified from isolated bovine heart mitochondria, and all the properties of this enzyme correspond to one of the forms isolated from the homogenate.

On the basis of these observations, it is concluded that malic enzyme is present in both the cytosol and the mitochondria of bovine heart, a distribution similar to that observed in bovine adrenal cortex but clearly distinct from that of liver.

Similarly, Hülsmann (16) reported the release of malic enzyme from sucrose-washed bovine heart sarcomeres.

This paper will present evidence which shows that two different forms of malic enzyme exist in bovine heart muscle. These two enzymes show clear differences in their chromatographic and electrophoretic properties and represent the activities located in the mitochondria and the cytosol of this tissue.

MATERIALS AND METHODS

Chemicals—NADP+, lactate dehydrogenase, malate dehydrogenase, and glucose 6-phosphate dehydrogenase were obtained from Boehringer Mannheim; L(-)-malic acid, 2-(N-morpholino)-ethanesulfonic acid, 2-(N-morpholino)-propanesulfonic acid, and DTT from Calbiochem; DEAE-cellulose and calcium phosphate gel from Bio-Rad, Richmond, California; NAD+, potassium pyruvate, NADH, glucose 6-phosphate, catalase, alcohol dehydrogenase, and β-mercaptoethanol from Sigma; and Sephadex and CM-Sephadex from Pharmacia.

The DEAE-cellulose employed was first washed extensively with 1 M Tris-Cl, pH 7.5, and then with distilled water. It was equilibrated with 30 mM Tris-Cl containing 1 mM β-mercaptoethanol, pH 7.5, and kept in this buffer in the cold.

Isolation of Bovine Heart Mitochondria—For the isolation of mitochondria, bovine hearts were processed immediately upon arrival from the slaughterhouse. After removal of adipose and connective tissues, the ventricular muscle was diced, rinsed with cold 0.25 M sucrose, and then ground with an electrically operated meat grinder. The ground muscle (1.5 kg) was suspended in 2.5 liters of 0.25 M sucrose, containing 1 mM potassium-EDTA, adjusted to pH 7.2 with KOH. The pH of the suspension was adjusted to 7.2 by the addition of 5 mM KOH, and the suspension was then homogenized in a continuous flow homogenizer, constructed as described by Ziegler and Pettit (17). The homogenate was subsequently fractionated by differential centrifugation as follows. Unbroken cells and debris were removed by centrifugation at 670 × g for 10 min, and the turbid supernatant fluid was decanted through two layers of cheesecloth. This filtrate was centrifuged at 13,000 × g for 15 min. The pellet was resuspended in sucrose-EDTA (approximately 0.1 volume of the original homogenate), and, after a brief homogenization with a loosely

1 The abbreviations used are: DTT, dithiothreitol; CM-Sephadex, CM-cellulose, carboxymethyl Sephadex and cellulose, respectively.

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Malic enzyme (malate:NADP+ oxidoreductase (decarboxylating), EC 1.1.1.40) was identified in avian liver by Ochoa and his collaborators (1-8). In all mammalian species studied so far, the malic enzymes have shown an absolute requirement for NADP+, as well as for a divalent cation, especially Mn2+ or Mg2+. The requirement for NADP+ is not true for microorganisms where both NAD+ and NADP+ linked activities have been demonstrated. The crystalline malic enzyme prepared from pigeon liver has been extensively studied by Lardy and his collaborators (9-12).

The intracellular localization of malic enzyme differs in the various tissues studied. Whereas the liver enzyme appears exclusively in the cytosol (10), its presence has been demonstrated in both the mitochondria and the cytosol in homogenates of rat brain (13) and bovine adrenal cortex (14). The work of Henderson (15) has indicated that in the case of heart muscle, the malic enzyme appears associated with the mitochondrial fraction.

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fitting Potter-Elvehjem homogenizer with a Teflon pestle, was centrifuged again for 15 min at 13,000 \( \times \) g. The washing procedure was repeated once more under identical conditions and the resulting pellet resuspended in a solution containing 5 mM \( \beta \)-mercaptoethanol (0.1 volume of the original homogenate). After stirring in the cold for 20 min, the suspension was again centrifuged at 13,000 \( \times \) g for 15 min. This wash removed the few red cells which contaminated the preparation at this point. The loosely packed mitochondria were finally resuspended in a solution containing 10 mM potassium phosphate, pH 7.4, and 5 mM \( \beta \)-mercaptoethanol, to a protein concentration of 15 to 20 mg per ml.

**Gel Electrophoresis**—Polyacrylamide gel electrophoresis was performed using the methodology of Davis (18). The separations were carried out in 7% gels at pH 8.3 at a current of 4 mA per gel. No sample gels were used, but the samples were mixed with an equal volume of 30% sucrose (w/v) and layered directly on top of the stacking gel. After electrophoresis, the gels were stained for proteins with a 1% solution of Amido black in 7% acetic acid and then dissolved in 3% NaOH before the addition of biuret reagent. Destaining was usually completed in 15 min. Identical gels were stained for enzymic activity by incubating the separated gels at room temperature and in the dark in a solution containing 2-(N-morpholino)-propanesulfonic acid (pH 7.5, MgCl\(_2\) (10 mM), NADP\(^+\) (0.33 mM), potassium malate (5 mM), phenazine methosulfate (0.1 mg per ml), and iodonitrotetrazolium violet (0.2 mg per ml). Activity blanks were incubated in the same solution without malate. Gels were stored in 7% acetic acid before photographing.

**Density Gradient Centrifugation**—Sucrose density gradient centrifugation was performed in a Beckman SW-60 rotor in an L2-65B Spinco ultracentrifuge. Three enzymes were used as molecular weight markers: malate dehydrogenase (mol wt, 60,000), catalase (mol wt, 240,000), and glucose 6-phosphate dehydrogenase (mol wt, 128,000). Fractions were collected from the top of the tubes by introducing a 45% sucrose solution at the saturation. After stirring in the cold for 30 to 45 min, the precipitated protein was separated by centrifugation at 13,000 \( \times \) g for 20 min and dissolved in 30 mM Tris-Cl-1 mM \( \beta \)-mercaptoethanol, pH 7.5, and dialyzed against 25 volumes of the same buffer. The dialyzed solution was further fractionated with ammonium sulfate as follows. Solid ammonium sulfate was added in the proportion of 51.6 g per liter of solution to give a 0.75 saturated solution with respect to the salt. After stirring for 1 hour, the precipitated protein was discarded after centrifugation for 20 min at 13,000 \( \times \) g. The supernatant solution was then adjusted to 0.75 saturation by the addition of 24.5 g of solid ammonium sulfate to each ml of supernatant. After stirring for 30 min in the cold, the precipitated protein was separated by centrifugation at 13,000 \( \times \) g for 20 min and dissolved in 30 mM Tris-Cl-1 mM \( \beta \)-mercaptoethanol, pH 7.5. The resulting solution was dialyzed overnight against 50 volumes of the same buffer.

**Chromatography on Sephadex G-100**

The dialyzed solution resulting from the first ammonium sulfate fractionation was chromatographed on a column (4 \( \times \) 75 cm) of Sephadex G-100, previously equilibrated with a buffer solution containing 30 mM Tris-Cl and 1 mM \( \beta \)-mercaptoethanol, pH 7.5. After the sample had been applied to the column, elution was performed with the same buffer until the enzyme activity eluted from the column. This procedure removed between 50 to 80% of the hemoglobin present in the protein fraction.

**Second Ammonium Sulfate Fractionation**

The active fractions eluted from the Sephadex column were pooled and fractionated with ammonium sulfate between 0.40 and 0.75 saturation, as indicated above. The fraction precipitating between these salt concentrations was dissolved in 3% NaOH before the addition of biuret reagent.
the protein and enzymic activity pattern obtained during this pH 7.5. This buffer caused the elution of a second peak of malic enzyme activity. The nomenclature used will be "Enzyme I" and "Enzyme II" to indicate the first and second peaks of activity emerging from DEAECellulose. Fig. 1 shows chromatography on DEAE-cellulose.

The dialyzed 0.4 to 0.75 fraction was applied to a column (5 x 40 cm) of DEAE-cellulose, prepared as indicated under "Materials and Methods." After the protein had entered the column, washing was started with 30 mM Tris-Cl-1 mM β-mercaptoethanol, pH 7.5. Fractions of 20 ml were collected and the activity assayed fluorometrically. The washing was continued until a large protein fraction, devoid of malic enzyme activity but containing isocitrate dehydrogenase activity, had emerged from the column. The elution buffer was then changed to 30 mM Tris-Cl-1 mM β-mercaptoethanol and 10 mM MgCl₂, adjusted to pH 7.5. Elution was continued until a second peak of protein, containing malic enzyme activity, had emerged from the column. Subsequently, the elution buffer was changed to 30 mM Tris-Cl-1 mM β-mercaptoethanol and 40 mM MgCl₂ at pH 7.5. This buffer caused the elution of a second peak of malic enzyme activity. The nomenclature used will be "Enzyme I" and "Enzyme II" to indicate the first and second peaks of activity emerging from DEAE-cellulose. Fig. 1 shows the protein and enzymic activity pattern obtained during this chromatography.

Treatment with Calcium Phosphate Gel

The fractions containing either Enzyme I or Enzyme II were pooled and then concentrated by precipitating the proteins with solid ammonium sulfate to a saturation of 0.75. The precipitated proteins were collected after centrifugation for 15 min at 20,000 x g and dissolved in a minimum volume of a buffer containing 30 mM Tris-Cl and 1 mM β-mercaptoethanol. The samples were dialyzed for 16 hours against 75 to 100 volumes of the same buffer. One volume of calcium phosphate gel (75 mg per ml) was added to the dialyzed preparations, and, after stirring in the cold for 10 min, the gel was separated by centrifugation at 5,000 x g for 5 min. All the activity of either Enzyme I or Enzyme II was absorbed to the gel under these conditions. Washing the calcium phosphate gel with 1 volume of 50 mm potassium phosphate buffer, pH 7.5, and stirring for 10 min removed approximately 10% of Enzyme II activity from the gel into the eluting medium. A second treatment of the gel with 1 volume of the same potassium phosphate buffer led to almost complete elution of this enzyme.

On the other hand, Enzyme I was not removed from the gel under these conditions. In order to elute the enzyme it was necessary to increase the concentration of potassium phosphate, pH 7.5, to 0.2 M, after which all the activity could be recovered in the supernatant fluid.

Chromatography on CM-Sephadex

The eluates from the calcium phosphate gel treatment were concentrated by saturating the solutions to 0.75 with ammonium sulfate (5.16 g of salt added per 10 ml of solution) and centrifugation for 15 min at 15,000 x g. The precipitated proteins were dissolved in a buffer containing 50 mM 2-(N-morpholino)ethanesulfonic acid, pH 6.5, and 10 mM β-mercaptoethanol and dialyzed against 100 volumes of the same buffer for 16 hours. The dialed preparations were applied to a column (1.5 x 40 cm) of CM-Sephadex C-50, previously equilibrated with dialysis buffer. The column was eluted stepwise with 100 ml of dialysis buffer and then with the same buffer to which 0.2 M KCl had been added. The initial wash removed a large protein peak, devoid of malic enzyme activity, whereas the elution with 0.2 M KCl eluted a small amount of protein which contained very high enzyme activity. The active fractions were pooled, the pH adjusted to 7.5 with 1 N KOH, and the solution saturated with ammonium sulfate to 0.75. After allowing 30 min, for equilibration, the precipitate was collected by centrifugation at 20,000 x g for 15 min and dissolved in a small volume of a buffer containing 100 mM 2-(N-morpholino)-propanesulfonic acid, pH 7.5, and 1 mM DTT.

Table I shows a summary of the purification procedure and the yield from a typical preparation. Further attempts to increase the specific activity of the final fractions, including chromatography on Sephadex G-100, preparative electrophoresis, and fractionation with ethanol or acetone have proven unsuccessful and resulted in large losses of activity.
Isolated bovine heart mitochondria, prepared as described under "Materials and Methods," demonstrated very little malic enzyme activity, even after being allowed to swell overnight at 4°C in 10 mM potassium phosphate at pH 7.4, and it was necessary to disrupt the organelles with the aid of sonic oscillation in order to release the activity into the soluble phase. As indicated in Table II, the release of malic enzyme from the mitochondrial preparation paralleled the increase of NADH oxidation activity observed in the same preparation. The mitochondria were, therefore, sonically oscillated for a total of 2 min (in 30-sec bursts) at a protein concentration of 7 to 10 mg per ml. Unbroken particles and fragments were removed by centrifugation at 40,000 × g for 45 min and the supernatant used for the purification of the mitochondrial malic enzyme, as described below.

**Purification of Mitochondrial Malic Enzyme**

Ammonium Sulfate Fractionation—Ammonium sulfate solution, saturated at 4°C and adjusted to pH 7.4 with NH₄OH, was added to the supernatant fluid obtained after sonic oscillation of the mitochondrial suspension in the ratio of 0.5 volume per volume of solution, to obtain a final saturation of 0.33 with respect to ammonium sulfate. After stirring at 4°C for 30 min, the solution was centrifuged for 15 min at 40,000 × g and the precipitate discarded. The supernatant fluid was then brought to 0.65 saturation by the slow addition of ammonium sulfate in the ratio of 2.14 g/10 ml of solution. The suspension was stirred for 30 min at 4°C and subsequently centrifuged for 15 min at 40,000 × g. The precipitate was dissolved in a solution containing 30 mM Tris-Cl, pH 7.5, and 0.1 mM DTT (approximately 0.1 volume per volume of the original mitochondrial suspension). The resulting solution was dialyzed overnight against 30 to 75 volumes of the same buffer.

**DEAE-cellulose Chromatography**—The dialyzed solution was centrifuged at 105,000 × g for 45 min in order to remove insoluble material and then applied to a column (2.5 × 40 cm) of DEAE-cellulose, prepared as described under "Materials and Methods" and eluted in the same manner as described previously for the separation of Enzyme I and Enzyme II from the complete homogenate. No trace of malic enzyme activity was detected until elution was carried out with 30 mM Tris-Cl containing 40 mM MgCl₂ at pH 7.5. This indicated the presence of only one of the two forms of the malic enzyme in the mitochondrial preparation. Fig. 2 shows the pattern obtained on DEAE-cellulose chromatography of the mitochondrial preparation.

**Gel Electrophoresis**

Samples of Enzyme I, Enzyme II, and mitochondrial malic enzyme were run simultaneously in analytical gel electrophoresis, by means of the procedure indicated under "Materials and Methods." Fig. 3 shows the results obtained after staining the gels for malic enzyme activity. Both Enzyme II and mitochondrial malic enzyme showed slightly greater mobility than Enzyme I. Each one of the three fractions tested yielded a single activity band, and Enzyme II appeared to be identical with the enzyme purified from the isolated mitochondria. As shown in the figure, mixtures of Enzyme I with either one of the other two purified fractions gave rise to two separate and clearly distinct activity bands. Blanks without malate did not produce

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**Table II**

**Release of Malic Enzyme from Isolated Heart Mitochondria**

Bovine heart mitochondria, prepared as described in the text, were treated as indicated. Aliquots of the treated samples were assayed fluorometrically for malic enzyme activity and NADH oxidation. Activities are expressed in μmoles per min per ml of mitochondrial suspension.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Malic enzyme</th>
<th>NADH oxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:5 dilution in 0.25 M sucrose</td>
<td>0.022</td>
<td>0.34</td>
</tr>
<tr>
<td>1:5 dilution in 5 mM mercaptoethanol</td>
<td>0.021</td>
<td>0.33</td>
</tr>
<tr>
<td>1:5 dilution in 10 mM phosphate + 5 mM mercaptoethanol</td>
<td>0.019</td>
<td>0.38</td>
</tr>
<tr>
<td>Same as above after 16 hours at 4°C</td>
<td>0.025</td>
<td>0.40</td>
</tr>
<tr>
<td>1:5 dilution in 10 mM phosphate + 5 mM mercaptoethanol and sonic oscillation for 2 min</td>
<td>0.124</td>
<td>2.8</td>
</tr>
</tbody>
</table>

**Fig. 2.** DEAE-cellulose chromatography of bovine heart mitochondrial malic enzyme. Fifteen-milliliter fractions were collected from a column, 2.5 × 45 cm, prepared as described under "Materials and Methods." Malic enzyme activity was measured fluorometrically. Optical density at 280 μm, ——; malic enzyme activity, • — •. For details see text.

The enzymes could be stored for 3 weeks at 4°C with a loss of activity of approximately 20%.

**Rechromatography of Enzymes I and II on DEAE-cellulose**

In order to establish with certainty whether the two fractions isolated from DEAE-cellulose chromatography represented two different forms of malic enzyme or were the result of a preparation artifact, the final isolated fractions were refractionated between 0.4 and 0.75 saturation with ammonium sulfate, dialyzed, and rechromatographed on DEAE-cellulose under conditions identical with those described above. The fractions appeared chromatographically homogeneous with respect to malic enzyme activity and only a single peak was observed for either Enzyme I or Enzyme II. This indicates the presence of two distinct forms of malic enzyme in bovine heart.
the reduction of the p-iodonitrotetrazolium used as an indicator, showing that the stain for enzymic activity was specific. The difference in mobility of the enzymes correlates well with the behavior observed during DEAE-cellulose chromatography, where Enzyme I is eluted first, indicating less negative charge than either Enzyme II or the mitochondrial malic enzyme.

**Stoichiometry of Reaction**

It was thought desirable to determine the stoichiometry of the reactions catalyzed by the two forms of malic enzyme in order to ascertain the nature of the reaction and to discard the possibility of other reactions that could cause the reduction of NADP⁺ in the presence of malate. As indicated in Table III, the ratio of pyruvate to NADPH is 1.0, which indicates the absence of other reactions that could cause the reduction of NADP⁺.

**Molecular Weight**

Two methods were employed in order to get an approximate molecular weight of the different malic enzymes at their present state of purity: density gradient centrifugation and gel filtration. Density gradient centrifugation was performed for 16 hours at 35,000 rpm in a 5 to 30% sucrose linear gradient containing 0.1 mM DTT. The centrifugation was performed in an L2-65B Spinco ultracentrifuge, in which the average centrifugal force was 120,000 x g. In all cases the malic enzyme sedimented between glucose 6-phosphate dehydrogenase and catalase. An approximate molecular weight of 200,000 ± 15,000 could be calculated for all three fractions tested (Enzyme I, Enzyme II, and mitochondrial enzyme).

The apparent molecular weight of the enzymes was also calculated by utilizing the method of Andrews (19) of filtration through a calibrated Sephadex column. Catalase, alcohol dehydrogenase, and crystalline human hemoglobin (courtesy of Dr. Michael Waterman) were used as marker proteins. The molecular weights obtained by this method were 195,000 for Enzyme I, 212,000 for Enzyme II, and 212,000 for the mitochondrial malic enzyme.

**Discussion**

Malic enzyme has been implicated as a possible important mediator in the transport of reducing equivalents from the cytosol to the mitochondria in adrenal cortex (22), as well as a source of NADPH for the mitochondrial hydroxylation of steroids in these same glands (23). The observation that the enzymic activity appeared elevated in the liver of mice under dietary conditions which favored lipogenesis (24) prompted Lardy et al. (25) to suggest that the role of malic enzyme in liver was to generate NADPH required for the synthesis of lipids. This suggestion was later confirmed by Young, Shrago, and Lardy (26), as well as by Wise and Ball (27). The suggestion by Hülsmann (16) that malic enzyme may play a role in the de novo synthesis of fatty acids in heart mitochondria still lacks experimental confirmation.

The intracellular localization of malic enzyme has been found to differ markedly in the different tissues so far studied. As mentioned previously, liver seems to contain exclusively a cytosol enzyme, whereas adrenal cortex and, apparently, brain possess two different forms of the enzyme. In adrenal cortex, one of the enzymes has been identified as a mitochondrial component. The experiments of Hülsmann (16) as well as Henderson (15) suggested that heart could possess a mitochondrial malic enzyme.

The results presented in this paper show clearly the presence of malic enzyme in both the cytosol and mitochondria of bovine heart. The appearance of a single form of the enzyme in preparations of mitochondria from this organ would make very unlikely
the possibility that the two forms isolated from the complete homogenate could result from a preparative artifact. As indicated above, no splitting of the activity was ever observed when any of the fractions isolated after DEAE-cellulose chromatography was refractionated with ammonium sulfate and rechromatographed on the same exchanger.

The behavior of the isolated enzymes on gel electrophoresis is also evidence of the different nature of Enzyme I and Enzyme II (which would be identical with the mitochondrial malic enzyme). The electrophoretic mobilities at pH 8.3 are fairly similar for all forms of the enzyme, but distinct enough to permit the observation of two different bands during the specific staining for malic enzyme activity. This pattern was obtained only in those cases in which Enzyme I and Enzyme II (or mitochondrial malic enzyme) were mixed and never when a single enzyme form was subjected to electrophoresis. It appears that Enzyme II is much more susceptible to inactivation during electrophoresis than Enzyme I. It is, therefore, important to notice the big difference in reactivity shown by the enzymes after the electrophoretic procedure. On every preparation, it has been observed that Enzyme II can be stained for activity on the gels by means of only a small fraction (0.05 to 0.025) of the units of activity necessary to detect that of Enzyme II. It appears that Enzyme II is much more susceptible to inactivation during electrophoresis than Enzyme I.

All the characteristics studies in this laboratory have shown identity between Enzyme II and the mitochondrial malic enzyme. The mitochondrial origin of this fraction is also apparent if the homogenization procedure is carried out in isotonic solutions, in which case the proportion of Enzyme II is considerably decreased with respect to that encountered in homogenates prepared in hypotonic solutions, as described in this paper.

Differences in kinetic behavior, sensitivity to sulfhydryl inhibitors, and other oxidizing agents have also been observed in preparations of Enzyme I and Enzyme II. It is, therefore, clear that malic enzyme is found in both the cytosol and the mitochondria of heart muscle and that the two forms of the enzyme have different properties.

The functional role of the two malic enzymes in heart muscle is only a matter of speculation at the present time, and further experiments will be required in order to establish their possible metabolic significance.

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REFERENCES

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