FACTORS AFFECTING THE ACTIVITY OF MITOCHONDRIAL AND SOLUBLE ACONITASE

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Considerable information on the intracellular localization of enzymes has been obtained in recent years through the technique of differential centrifugation of sucrose homogenates of tissues and subsequent testing of the fractions for enzyme activity. One of the results of this work has been the demonstration that rat liver mitochondria (Mw) are capable of completely oxidizing a wide variety of substrates by way of the reactions of the tricarboxylic acid cycle (1). Consequently, mitochondria should contain an active aconitase. This supposition has been verified. The aconitase associated with mitochondria is released into solution after freezing and thawing. It therefore became possible to compare the properties of the particulate-bound enzyme with its released (soluble) counterpart.1

A number of interesting points have been brought out by this comparison. Some factor, presumably the mitochondrial membrane, is responsible for a difference between the pH optimum of the particulate aconitase and that of the soluble enzyme. It has also been observed that appreciable amounts of aconitate (AA) are retained within the particle in the conversion of isocitrate (ICA) to citrate (CA).

These differences in the activity of mitochondrial aconitase, which probably are not directly related to the properties of the protein itself, point up the necessity of considering permeability factors in the measurement of particle-bound enzymes. Thus the data on the intracellular distribution of aconitase have been found to be markedly affected by the pH of the assay medium. The results invalidate the assumption that the physical environment of an enzyme has no effect on its relative activity under varying assay conditions.

Methods and Materials

Substrates—cis-Aconitic anhydride was prepared from trans-aconitic acid by the procedure of Malachowski and Maslowski (2). It was recrystallized

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1 The solubility status of the mitochondria-bound aconitase is not known. It is not soluble in the usual sense of this term. In this paper, therefore, the enzyme released from mitochondria by freezing and thawing will be termed the soluble enzyme, as distinguished from the mitochondria- or particulate-bound enzyme.
from benzene, m.p. 77°. \( dl \)-Isocitric lactone was synthesized by the procedure of Kato and Dickman (3), m.p. 160-161°, and was converted to free isocitrate by the procedure of Krebs and Eggleston (4). The \( \alpha \)-, \( \beta \)-, and \( \gamma \)-monomethyl esters\(^2\) of \( cis \)-aconitic acid were synthesized by the procedures of Malachowski et al. (5).

Analytical Methods Citric acid was determined by the procedure of Dickman and Cloutier (6), except that HPO\(_3\) was used as the deproteinizing agent (7). 0.2 ml. of KMnO\(_4\) was required in the oxidation of solutions which contained sucrose, and 0.4 ml. was required by solutions which contained both sucrose and aconitate.

Aconitic acid was determined by the method of Dickman (8). Since tissue extracts commonly reduce KMnO\(_4\) to a slight degree, inactive enzyme controls were routinely run by the addition of HPO\(_3\) to the substrate solution before the addition of enzyme. The absorbancy of this solution at 530 m\( \mu \) was used in the calculation of the aconitate formed by the active enzyme.

Differential Centrifugation of Rat Liver Homogenates—Adult rats, 200-300 gm. (Sprague-Dawley), were decapitated. The livers were immediately placed in cold 0.25 M sucrose, cut into small pieces, and stirred for a few minutes in cold sucrose, then drained and homogenized in cold 0.25 M sucrose in 2 gm. portions. Differential centrifugation followed the procedure of Schneider and Hogeboom (9), except that the small particle fraction was centrifuged for 15 minutes at 21,000 \( \times g \).

The fluffy layer above the mitochondria was removed with a side hole pipette and added to the supernatant solution.\(^3\) Each fraction was washed twice with the sucrose solution.

Release of Mitochondrial Aconitase—The aconitase associated with the mitochondria could not be removed by repeated washings with cold 0.25 M sucrose. The enzyme could be separated from the particles, however, by freezing and thawing. Mitochondrial suspensions in 0.25 M sucrose were frozen and thawed, then centrifuged at 21,000 \( \times g \) for 30 minutes at 5°. The clear, faintly yellow supernatant solution contained an active aconitase (Sol\( _R \)). It was found that the time of freezing influenced the amount of aconitase which was released. Less aconitase became soluble when mitochondria were frozen and thawed three times in 2 hours than when they remained at \(-20°\) for 2 to 10 days before thawing.

Results

Measurement of Aconitase Activity—Since mitochondria are capable of the complete oxidation of tricarboxylic acids under aerobic conditions,

\(^2\) These compounds were synthesized by H. P. Kato.

\(^3\) Recknagel, R. O., personal communication.
aconitase assays were routinely carried out in evacuated Thunberg tubes. In order to avoid disintegration of Mw during the incubation, the assay medium was adjusted to an isotonic concentration with sucrose solution. Each tube contained 1.2 ml. of 0.1 M dl-isocitrate, citrate, or aconitate, pH 7.2, 0.6 ml. of 0.25 M sucrose, and 0.7 ml. of water. 0.5 ml. of the enzyme preparation in sucrose was placed in the cap. The tubes were incubated for 10 minutes at 30°, and the reaction was stopped by the addition of 0.5 ml. of fresh 30 per cent HPO₃. The precipitate was removed by centrifugation and analyses were carried out on aliquots of the supernatant solution. Unless otherwise specified, these standard conditions were main-

\[\text{Fig. 1. Enzyme concentration curves of aconitase. Curve I, ICA } \xrightarrow{\text{Solp}} \text{ AA; Curve II, AA } \xrightarrow{\text{Mw}} \text{ CA; Curve III, ICA } \xrightarrow{\text{Mw}} \text{ AA; Curve IV, ICA } \xrightarrow{\text{Mw}} \text{ CA; Curve V, CA } \xrightarrow{\text{Mw}} \text{ AA.}\]

Fig. 2. Relative activities of mitochondrial and soluble aconitase with different substrates. Assay conditions described in the text.

Relative Activities of Mitochondrial and Soluble Aconitase in Tricarboxylic Acid Interconversions—A fresh mitochondrial suspension in 0.25 M sucrose was tested for aconitase activity. A portion of the suspension was stored at -20° for 10 days, then thawed and centrifuged, and a comparison of the activities of the two preparations with all three substrates was made. As shown in Fig. 2, both the mitochondrial and soluble aconitase formed more AA from ICA than from CA. Similar data, obtained with a soluble aconitate, have been reported by Racker (10).

In the one-step reactions, ICA → AA and CA → AA, a marked increase
in total activity was observed on release of the enzyme. In the ICA →
CA conversion, a two-step reaction, the soluble enzyme was less active
than the mitochondria from which it was derived. Any activators re-
leased from the mitochondria should have affected the soluble enzyme in a
consistent manner. It therefore appeared that the anomalous results cited
above were related to the properties of the mitochondria themselves, which
in turn were affecting the activity of the bound aconitase. The fact that
mitochondria were more efficient than the soluble enzyme in the two-step
reaction raised the possibility that the aconitate formed from ICA was
being held within the membrane, thus effectively raising the substrate con-
centration in the subsequent reaction to citrate.

A suspension of intact mitochondria was incubated with ICA in isotonic
media in two separate tubes. At the end of 10 minutes each tube was chilled

<table>
<thead>
<tr>
<th>Treatment of mitochondria</th>
<th>Aconitate per 3.5 ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
</tr>
<tr>
<td>Centrifuged</td>
<td>0.57</td>
</tr>
<tr>
<td>Lysed</td>
<td>1.00</td>
</tr>
</tbody>
</table>

and centrifuged at 0° for 5 minutes at 18,000 \( \times g \). An aliquot of the super-
натant solution from one tube was added to one-sixth volume of 30 per
cent HPO\(_3\). The entire contents of the other tube were well mixed with a
plunger and one-sixth volume of 30 per cent HPO\(_3\) was added. Both tubes
were centrifuged and the aliquots analyzed for AA. Table I includes the
results of three experiments. The data show that mitochondria disrupted
with HPO\(_3\) released more AA into the solution than was present in the
supernatant solution from which the mitochondria had previously been re-
moved. This result is consistent with the explanation given above.

**pH-Activity Curves of Mitochondrial and Soluble Aconitase**—Recent data
(11, 12) indicate that mitochondria are organized systems surrounded by
a membrane. It is also known that the permeability of semipermeable
membranes is markedly affected by pH (13). Mitochondria were sus-
pended in isotonic solutions of varying pH values and their aconitase ac-
tivities were determined. Similar determinations were carried out with
the released aconitase. As shown in Fig. 3, a biphasic activity curve was
obtained with mitochondria. At pH 5.8 they converted more ICA to AA
than at pH 7.3. Similarly the conversion of ICA to CA shows a peak at pH 5.8, although it is lower than at pH 7.3. In contrast to these curves, the soluble enzyme has very low activity at pH 5.8, and its activity at pH 7.3 is about 8 times as great as at the more acid pH.

These differences in activity of the soluble and particulate aconitase as influenced by pH may be ascribed to (1) the physical state of the enzyme altering its pH optimum or to (2) the permeability of the mitochondrial membrane affecting the amount of substrate which comes in contact with the enzyme. While the pH optimum of a number of soluble enzymes is known to be affected by such factors as ionic strength, type of buffer, or specific substrate, we are unaware of any work which indicates that physical status per se influences this property.

It therefore seems probable that the mitochondrial membrane is more permeable to ICA at pH 5.8 than at pH 7.3. The results with the mitochondria also suggest that the higher AA:CA ratio at pH 5.8 compared to that at pH 7.3 is due to slower diffusion of the aconitate which is formed in the reaction at the latter pH.

**Inhibition of Aconitase by o-Phenanthroline and Reactivation by Ferrous Ions**—When aconitase from heart muscle was incubated with \( \alpha, \alpha'- \text{bipyridyl} \) or o-phenanthroline, an inhibition of the AA \( \rightarrow \) CA reaction was observed. This inhibition could be reversed by the addition of ferrous ions (6). Similar experiments have been carried out with liver mitochondria and the

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**Fig. 3.** pH-activity curves of mitochondrial and soluble aconitase. Curve I, ICA \( \text{MW} \rightarrow \text{AA} \); Curve II, ICA \( \text{MW} \rightarrow \text{CA} \); Curve III, ICA \( \text{Solp} \rightarrow \text{AA} \); Curve IV, ICA \( \text{Solp} \rightarrow \text{CA} \). pH adjusted by the addition of \( \text{n HCl} \) to isotonic solutions containing 0.04 \( \text{m isocitrate solution, pH 7.3} \).
soluble aconitase derived from them. The data of representative experiments are shown in Fig. 4. Aconitase was inhibited by o-phenanthroline whether the enzyme was associated with intact mitochondria or was in solution. Partial reactivations of inhibited aconitase are demonstrated in both the ICA → AA and CA → AA reactions as well as the stabilizing effect of Fe++ plus ascorbate on the uninhibited enzyme. The data are consistent with the view that aconitase is a single entity. On the other hand, the possibility that aconitase consists of two proteins, each activated by Fe++, is not excluded by these results. These results confirm and extend the previous data obtained with aconitase from heart.

Intracellular Distribution—The aconitase activities of the various intracellular fractions with 0.04 M ICA as substrate are set out in Table II. At pH 7.3 the bulk of the activity was found in the soluble fraction. When the aconitase activity of the homogenate and various intracellular fractions was determined at pH 5.8, a significantly different distribution of activities was found (Table II, third and fourth columns). At pH 5.8 the mitochondrial fraction exhibited a higher percentage of the total aconitase activity than did the supernatant fraction. It is obvious from the data of Fig. 3 that, if the ICA → CA reaction at pH 5.8 or pH 7.3 had been used as the criterion of aconitase activity, still other distributions of activity would have been found. The data on the intracellular distribution
of aconitase are therefore dependent on the conditions of the assay. This conclusion may also apply to other enzymes.

Substrate Specificity of Aconitase—The three isomeric monomethyl esters of cis-aconitate were tested as aconitase substrates with a soluble enzyme. The KMnO₄ reaction was utilized to determine whether a decrease in the concentration of unsaturated material had occurred. No evidence of a reaction was found. The esters also appeared to be inert as inhibitors of the enzyme. When equal concentrations of each ester and AA were incubated with aconitase, the amount of citrate formed was the same in all tubes. These results demonstrate that all three carboxyl groups must be free in cis-aconitate for the hydration reaction to occur.

Table II

Intracellular Distribution of Aconitase

Assays were run under standard conditions with dl-isocitrate substrate. Solutions were adjusted to pH 7.3 or 5.8 with n HCl.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Aconitate formed per gm liver, pH 7.3</th>
<th>Relative activity, pH 7.3</th>
<th>Aconitate formed per gm liver, pH 5.8</th>
<th>Relative activity, pH 5.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>40</td>
<td>100</td>
<td>15</td>
<td>100</td>
</tr>
<tr>
<td>Nuclei</td>
<td>3.2</td>
<td>8</td>
<td>3.2</td>
<td>14</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>6.5</td>
<td>16</td>
<td>7</td>
<td>45</td>
</tr>
<tr>
<td>Particles</td>
<td>0.6</td>
<td>1.4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Supernatant</td>
<td>38</td>
<td>95</td>
<td>5</td>
<td>33</td>
</tr>
</tbody>
</table>

Discussion

The effects of the mitochondrial membrane on the activity of particulate enzymes are gradually becoming recognized. Lehninger (14) has found that the rate of oxidation of reduced coenzyme I by rat liver mitochondria is strongly influenced by the pretreatment of the particles. He interpreted the results in terms of permeability effects. Similarly, Schneider et al. (15) observed that the medium in which liver is homogenized profoundly affects both the activity of cytochrome c and retention of cytochrome c in the mitochondria. Whether these results have a direct connection with the “exogenous” and “endogenous” cytochrome c activity of succinoxidase preparations reported by Keilin and Hartree (16), Tsou (17), and Slater (18) is not clear at present. Berthet et al. (19) have found that the acid phosphatase of mitochondria is inactive toward glycerophosphate until the particles are disrupted. They suggested that the mitochondrial membrane prevented contact of substrate and enzyme. Crane and Lipmann (20) recently reported that mitochondrial inorganic phosphate exchanged more
slowly with that in the medium than did adenosinetriphosphate. They postulated that a diffusion barrier to inorganic phosphate was present in the mitochondria.

The effect of pH on the activity of particulate enzymes has received little attention to date. Huennekens (21) has reported that the pH optimum of the malic dehydrogenase of the cyclophorase preparation was in the range pH 7 to 8, while that of the soluble enzyme was approximately pH 9.5. The separation of aconitase from mitochondria provides clear cut evidence that the disruption of the mitochondrial structure releases an enzyme with a different pH-activity spectrum from that of the intact particles. The pH optima of other soluble enzymes may be quite different from that found when the enzyme is enclosed within a semipermeable membrane. Another question concerns the physical status of mitochondria-bound enzymes; i.e., are they present in solution inside the mitochondria or are they present as part of an organized, insoluble matrix? The data are at present insufficient to decide between these alternatives.

These results make the interpretation of intracellular enzyme distribution studies more difficult. With aconitase, and probably with other enzymes as well, the relative activity of the various fractions is in large part determined by the assay conditions. When an enzyme activity is found to be present in more than one fraction of a homogenate, it may be necessary to obtain complete release of the particulate-bound enzyme into solution. If this is accomplished, variations in membrane permeability due to pH or other factors will not influence the activity of the enzyme under investigation and thus affect the calculation of intracellular enzyme distribution.

**SUMMARY**

Aconitase has been prepared from mitochondria by freezing and thawing. The soluble aconitase is more active than the mitochondrial enzyme in the isocitrate-aconitate and citrate-aconitate reactions, but mitochondrial aconitase is more active in the isocitrate-citrate transformation. Soluble aconitase showed an optimum at pH 7.3 but mitochondrial aconitase showed optima at pH 5.8 and 7.3.

The isocitrate-aconitate and citrate-aconitate reactions of both soluble and mitochondrial aconitase were inhibited by o-phenanthroline and reactivated by ferrous ions plus ascorbate.

Mitochondria incubated with isocitrate retain aconitase. The data on the intracellular distribution of aconitase were markedly affected by the conditions of the assay.

The three monomethyl esters of aconitate function neither as substrates nor as inhibitors of aconitase.
BIBLIOGRAPHY

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Sherman R. Dickman and Joseph F. Speyer