Mitochondrial Isocitrate Dehydrogenases from Yeast*

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SUMMARY

A polarographic technique suitable for kinetic studies of either isolated or bound pyridine nucleotide-linked dehydrogenases was used to investigate the isocitrate dehydrogenases of yeast mitochondria. Intact mitochondria were found to contain both triphosphopyridine nucleotide- and diphosphopyridine nucleotide-linked isocitrate dehydrogenases with properties very similar to those which have been reported for the isolated dehydrogenases. The DPN-linked enzyme in situ exhibits a nonclassical, sigmoid substrate against activity relationship with respect to isocitrate and is activated by adenosine 5'-monophosphate. The TPN-linked enzyme in situ exhibits a hyperbolic substrate against activity relationship which is unaffected by AMP.

The DPN-linked dehydrogenase also shows another nonclassical increase in activity that occurs in response to high isocitrate concentrations or to heat treatment. This activation is characteristic only of untreated, intact mitochondria and may be secondary to an alteration of the mitochondria themselves.

The oxidation of three-n-isocitrate to α-ketoglutarate and CO₂ was first shown by Kornberg and Pricer (1) to proceed via two separate pathways in yeast, one mediated by a TPN-linked dehydrogenase and the other by a DPN-linked dehydrogenase. These two enzymes differ from each other in a number of significant features, which are undoubtedly related to the distinct physiological roles they fulfill.

TPN-linked isocitrate dehydrogenase is predominantly a “soluble” enzyme, and only a small proportion is localized in mitochondria (2, 3). Its activity is freely reversible, and the enzyme exhibits classical Michaelis-Menten kinetic behavior (1). DPN-linked isocitrate dehydrogenase, on the other hand, is associated exclusively with mitochondria (4), does not readily catalyze the reductive carboxylation of α-ketoglutarate (1, 5, 6), and possesses unusual kinetic properties. The substrate against activity relationship of the DPN-linked dehydrogenase is sigmoid rather than hyperbolic with respect to isocitrate, and the enzyme from plant sources (5, 7) is specifically activated by AMP, and that from animal sources (8, 9) by ADP.

The activities of these enzymes and the factors which influence them are of fundamental importance in the functioning of the Kreb’s cycle and in the over-all integration of cellular isocitrate utilization. Most of the characterization studies of the isocitrate dehydrogenases have been conducted with the isolated or purified proteins; however, it is conceivable that isolation of the dehydrogenases, particularly from organelles such as mitochondria, may be accompanied by some alteration in structure and catalytic properties. Since it is important to establish the behavior of these enzymes prior to the application of any technique which may possibly alter them, an attempt was made to study the isocitrate dehydrogenases directly in intact mitochondria. A polarographic procedure for measuring the activity of these enzymes has proven to be very useful for this purpose.

METHODS AND MATERIALS

Growth of Yeast Cells—A strain of Saccharomyces cerevisiae isolated from commercial yeast (Red Star Yeast and Products) was grown aerobically for 12 hours according to the procedure outlined by Duell, Inoue, and Utter (10). After the cells were harvested by centrifugation, they were suspended in water, filtered and washed on Millipore filters (RA-47), and stored as a cake at 4° until used (generally the next day).

Preparation of Mitochondria—Intact mitochondria were obtained by the osmotic shock of spheroplasts derived from 30 g (wet weight) of yeast by the two-step method described by Duell et al. (10). Six different preparations were used for the studies reported in the present paper. Suspensions containing about 1% of mitochondrial protein in a medium of 20% sucrose (w/v), 1 mM disodium EDTA, and 50 mM potassium phosphate (pH 6.8), were rapidly frozen with an ethyl Cellulose-Dry Ice mixture and kept at −30° until used.

Freezing itself does not affect the catalytic activity of the bound isocitrate dehydrogenases, and frozen mitochondria show no loss of isocitrate dehydrogenase activity on storage for up to 4 months. Intact yeast mitochondria are relatively resistant to the effects of freezing, and only small decreases in major oxidative functions are induced by this treatment. Frozen yeast mitochondria, for example, will exhibit a high respiratory control ratio in association with the oxidation of α-ketoglutarate. Their stability is further illustrated by the finding of Ohnishi, Kawaguchi, and Hagihara (11) that storage of yeast mitochondria at 0° for 5 days does not alter the respiratory control of α-ketoglutarate oxidation, although there is a decline of the P:O ratio.

Heated Mitochondria—Unless otherwise stated, heated mito-

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1 E. A. Duell, P. J. Russell, Jr., and M. F. Utter, unpublished observations.
Mitochondria were prepared by maintaining a suspension as above at 55° for 15 min. The mitochondria were then chilled in ice, diluted with an equal volume of the suspending medium described above, centrifuged at 10,000 × g for 10 min, and finally resuspended and either used directly, or frozen as above.

**Oxygen Uptake**—Dissolved oxygen was measured polarographically with a Clark oxygen electrode (Yellow Springs Instrument Company) polarized at −0.6 volt. The electrode was adapted to fit a Plexiglas reaction chamber of 2-ml capacity which was equipped with magnetic stirring. Recordings were obtained with a 0- to 5-mv Leeds and Northrup model H strip-chart recorder.

All assays were conducted at 30° in a final volume of 1.5 ml. About 3 to 4 min were required for temperature equilibration, and aluminum foil was used to darken the chamber when phenazinium salts were present. Reactions were started by the addition of mitochondria through a small side arm which could be plugged. Calculations of oxygen uptake are based on a dissolved oxygen concentration of 0.240 mM. Specific activity is defined as the micromoles of O2 taken up per min per mg of mitochondrial protein.

**Chemicals**—Phenazine ethosulfate was synthesized according to Moillain (12) and recrystallized several times from ethanol. The spectral properties of this compound were found to be the same as those reported for phenazinemethosulfate (13). Phenazine ethosulfate was preferred over phenazinemethosulfate because it is less inhibitory in certain instances and is more stable at higher pH values. Aqueous stock solutions were stored at room temperature in absolute darkness. DPN was the product of Boehringer. TPN, Tris, trisodium threo-β-α-ketoglutarate, and AMP were obtained from Sigma. Bovine serum albumin was the product of Pentex. Unless otherwise stated, Fraction V BSA was used. The term "isocitrate" in this paper always refers to the concentration of threo-β-α-ketoglutarate (14), even though the mixture of threo isomers was actually used.

Protein was determined according to Lowry et al. (15) with crystalline BSA as a standard. Glass-distilled water was used for the preparation of all reagents.

**Assay of Mitochondrial Isocitrate Dehydrogenase**—The measurement of isocitrate dehydrogenase activity is based on the rapid oxidation of reduced pyridine nucleotide by phenazine ethosulfate and the subsequent reoxidation of the reduced phenazinium salt by oxygen (16-18). The stoichiometry of this reaction was verified by a direct comparison of the oxygen consumed and the hydrogen peroxide produced after a phenazinium salt by oxygen (16-18). The stoichiometry of this reaction was verified by a direct comparison of the oxygen consumed and the hydrogen peroxide produced after a phenazinium salt by oxygen (16-18). The stoichiometry of this reaction was verified by a direct comparison of the oxygen consumed and the hydrogen peroxide produced after a phenazinium salt by oxygen (16-18). The stoichiometry of this reaction was verified by a direct comparison of the oxygen consumed and the hydrogen peroxide produced after a phenazinium salt by oxygen (16-18). The stoichiometry of this reaction was verified by a direct comparison of the oxygen consumed and the hydrogen peroxide produced after a phenazinium salt by oxygen (16-18). The stoichiometry of this reaction was verified by a direct comparison of the oxygen consumed and the hydrogen peroxide produced after a phenazinium salt by oxygen (16-18). The stoichiometry of this reaction was verified by a direct comparison of the oxygen consumed and the hydrogen peroxide produced after a phenazinium salt by oxygen (16-18). The stoichiometry of this reaction was verified by a direct comparison of the oxygen consumed and the hydrogen peroxide produced after a phenazinium salt by oxygen (16-18). The stoichiometry of this reaction was verified by a direct comparison of the oxygen consumed and the hydrogen peroxide produced after a phenazinium salt by oxygen (16-18). The stoichiometry of this reaction was verified by a direct comparison of the oxygen consumed and the hydrogen peroxide produced after a phenazinium salt by oxygen (16-18). The stoichiometry of this reaction was verified by a direct comparison of the oxygen consumed and the hydrogen peroxide produced after a phenazinium salt by oxygen (16-18). The stoichiometry of this reaction was verified by a direct comparison of the oxygen consumed and the hydrogen peroxide produced after a phenazinium salt by oxygen (16-18). The stoichiometry of this reaction was verified by a direct comparison of the oxygen consumed and the hydrogen peroxide produced after a phenazinium salt by oxygen (16-18). The stoichiometry of this reaction was verified by a direct comparison of the oxygen consumed and the hydrogen peroxide produced after a phenazinium salt by oxygen (16-18).

**RESULTS**

*Demonstration of Mitochondrial TPN- and DPN-linked Isocitrate Dehydrogenases and Effect of AMP*—The polarograms shown in Fig. 3 demonstrate the presence of both the TPN- and DPN-linked isocitrate dehydrogenases in mitochondria. When the initial medium is supplemented with TPN, oxygen uptake commences immediately with the addition of isocitrate, and the rate of this reaction is not increased by subsequent addition of AMP. When the initial medium is supplemented with DPN, however, there is only a small oxygen uptake in response to isocitrate, and subsequent addition of AMP results in a marked stimulation of activity. Kornberg and Price (1) first showed that AMP behaves as an activator of the DPN-linked isocitrate dehydrogenase, but not of the TPN-linked enzyme from yeast. The present experiment shows that these features can be shown to operate in intact mitochondria, and it further indicates that a kinetic approach may be used for the investigation of these enzymes in situ. The rates shown in
Fig. 3 do not reflect the true relative activities of the dehydrogenases, because of the low level of isocitrate used.

**Relative Activities of Mitochondrial TPN- and DPN-linked Isocitrate Dehydrogenases**—The specific activities of the two mitochondrial dehydrogenases were obtained (Table I) with elevated substrate concentrations in order to assay the enzymes near their maximal velocities. The exact determination of these activities is complicated by the fact that the mitochondria contain some bound pyridine nucleotide. However, as will be pointed out below, much of this appears to be in the form of DPN which can be largely removed by heating the mitochondria. The best values for the specific activities of the TPN enzyme are probably given by the differences between Lines 3 and 1 of Table I. Thus, the specific activity is about 0.2 to 0.3 for the TPN enzyme, and about 0.7 for the DPN enzyme. Although the activities observed with TPN and DPN combined are always larger than those with TPN or DPN alone (Table I), they are never equal to the sum of the activities of the separate TPN- and DPN-linked enzymes.

**Depression of Pyridine Nucleotides from Mitochondria and Dependence of Isocitrate Dehydrogenases on TPN and DPN**—When untreated mitochondria are assayed at high isocitrate concentration in a medium unsupplemented with pyridine nucleotide, appreciable activity is seen to be present. This activity is rapidly lost if the mitochondria are preincubated in the absence of substrate. Fig. 4 shows the effect of preincubating a mitochondrial suspension for various lengths of time and at different temperatures on the dehydrogenase activity observed in the absence of added pyridine nucleotide. The decreases which are seen presumably reflect only the loss of bound coenzyme, because the heated mitochondria assayed in the presence of added DPN show no decrease in activity. The reduction of endogenous activity by means of heat closely resembles the depletion of pyridine nucleotides from rat liver mitochondria (19-21).

With the use of heated mitochondria, the pyridine nucleotide dependencies of the isocitrate dehydrogenases were examined. Fig. 5 shows that the DPN-linked enzyme has an apparent \( K_m \) of 2.6 \( \times 10^{-4} \) m for DPN, and Fig. 6 shows that the TPN-linked enzyme has an apparent \( K_m \) of 2.2 \( \times 10^{-5} \) m for TPN.

**Table I**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Specific activity of mitochondria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
</tr>
<tr>
<td>No added coenzyme</td>
<td>0.229</td>
</tr>
<tr>
<td>With DPN</td>
<td>0.666</td>
</tr>
<tr>
<td>With TPN</td>
<td>0.493</td>
</tr>
<tr>
<td>With DPN + TPN</td>
<td>0.778</td>
</tr>
</tbody>
</table>

FIG. 1. Dependence of mitochondrial DPN-linked isocitrate dehydrogenase on phenazine ethosulfate. Assays contained: 0.1 m Tris-chloride, pH 8.0; 0.2% BSA; 6.67 \( \times 10^{-5} \) m DPN; 5.0 \( \times 10^{-4} \) m KCN; 6.67 \( \times 10^{-5} \) m phenazine ethosulfate; and 1.67 \( \times 10^{-3} \) m TPN or DPN. Additions: 0.375 mg of mitochondrial protein, 3.33 \( \times 10^{-4} \) m isocitrate, and 6.67 \( \times 10^{-3} \) m AMP. Concentrations are based on a final volume of 1.5 ml. The use of crystalline BSA in place of Fraction V albumin is necessary in order to obtain the low rate of "endogenous" activity seen prior to the addition of substrate. The numbers in parentheses represent the uptake of dissolved oxygen in millimicromoles per min. See Table I for the specific activities of the dehydrogenases at high concentrations of isocitrate.

Fig. 2. Proportionality of mitochondrial DPN-linked isocitrate dehydrogenase activity to concentration of mitochondrial protein. Assays contained: 0.1 m Tris-chloride, pH 8.0; 0.2% BSA; 6.67 \( \times 10^{-5} \) m MgC\(_2\); 6.67 \( \times 10^{-4} \) m DPN; 5.0 \( \times 10^{-4} \) m KCN; 3.33 \( \times 10^{-4} \) m AMP; 6.67 \( \times 10^{-3} \) m isocitrate; 4.67 \( \times 10^{-3} \) m phenazine ethosulfate; and mitochondrial protein as indicated.
Mitochondrial Isocitrate Dehydrogenases

The apparent \( K_m \) of \( 5.8 \times 10^{-4} \) M, determined in the presence of 3.3 mM TPN, is not altered by the presence of 0.30 mM AMP. This \( K_m \) is comparable to the value of \( 4.0 \times 10^{-5} \) M reported by Kornberg and Pricer (1) for the purified enzyme in the presence of 2.3 mM TPN. It should be emphasized that the \( K_m \) values given here are valid only for the fixed conditions specified and are intended to serve only as a guide to the catalytic requirements of the enzymes.

Dependence of Mitochondrial DPN-linked Isocitrate Dehydrogenase on AMP and Isocitrate—The activating effect of AMP on the mitochondrial DPN-linked isocitrate dehydrogenase is illustrated in Fig. 3. This effect is highly dependent on the concentration of isocitrate. As shown by the results in Fig. 8, the activity is seen to increase in a sigmoid fashion with increasing concentrations of isocitrate. At concentrations of 0.2 mM or less, no measurable activity is observed in the absence of AMP. This is in agreement with the findings reported by Hathaway and Atkinson for the purified enzyme (5).

For any given concentration of isocitrate (and at fixed levels of Mg\(^{2+}\) and DPN), the dependence of the activity on the AMP concentration can be determined and an apparent \( K_m \) computed. The dependence of the reaction on AMP at an isocitrate concentration of 0.5 mM is shown in Fig. 9. Under the conditions specified, the apparent \( K_m \) for AMP is seen to be \( 3.9 \times 10^{-5} \) M. By using an isocitrate concentration of 0.08 mM, Kornberg and Pricer (1) reported an apparent \( K_m \) of \( 0.9 \times 10^{-5} \) M for AMP with the purified DPN-linked enzyme from yeast.

The dependence of the mitochondrial DPN-linked enzyme on isocitrate actually reveals two types of nonclassical dependence upon substrate. With untreated mitochondria (see Fig. 10),
the dependence of the activity on isocitrate gives rise to a double reciprocal plot which at low concentrations of substrate curves sharply upward in the absence of AMP but is linear in the presence of 0.5 mM AMP. At higher concentrations of isocitrate, the activating effect of AMP decreases until it is no longer evident. Although no dependence on AMP is apparent in this experiment at isocitrate concentrations of 0.667 mM and greater, it should be pointed out that this effect is not always obtained, and in other experiments it was possible to observe an effect by AMP at all concentrations of isocitrate, with the double reciprocal plots intersecting at $V_{\text{max}}$. The cases in which added AMP does not show an effect at higher isocitrate concentrations may possibly be explained by the presence of AMP either in the mitochondrial preparation or as an impurity in a reagent.

The second type of nonclassical dependence upon substrate is seen with untreated mitochondria at high concentrations of isocitrate, where there is an unexpected increase in activity which results in a downward deflection of the double reciprocal plot (see Fig. 10). When heated mitochondria are used, not only is there a greater dependence on AMP at all concentrations of isocitrate, but in addition, the downward deflection of the double reciprocal plot at high concentrations of isocitrate is removed. A comparison of the isocitrate dependencies with heated and untreated mitochondria in the presence or absence of AMP shows that there is no change in the $V_{\text{max}}$ of the double reciprocal plots. It should be realized that it was not possible to examine this second type of activation with the mitochondrial TPN-linked isocitrate dehydrogenase, because this enzyme can be tested only in DPN-depleted (e.g. heated) mitochondria.

**DISCUSSION**

In this paper, direct evidence is provided for the existence of both the TPN- and DPN-linked pathways of isocitrate oxidation in yeast mitochondria. The fact that the mitochondrial DPN-linked enzyme is activated by isocitrate and AMP indicates that these properties are not unique to the purified enzyme, but are indeed characteristic of the dehydrogenase in situ. This is an important point to establish, as it is conceivable that alterations in the structure and therefore also in the catalytic properties of the dehydrogenase could result from isolation of the enzyme from its mitochondrial environment. The present results supplement the survey made by Goebell and Klingenberg (9), who extracted and assayed the two isocitrate dehydrogenases from the mitochondria of various animal tissues. These authors showed that the DPN-linked enzyme always occurs in a fixed ratio with regard to the cytochrome a content of the mitochondria, whereas the TPN-linked enzyme is present in amounts which may be either greater or less than that of the DPN-linked enzyme.

This study shows that with untreated mitochondria in the
absence of AMP, there is no detectable reaction, with or without added DPN, at isocitrate concentrations of up to 0.2 mM. Inasmuch as this concentration is about 3.5-fold the $K_a$ for isocitrate of the TPN-linked enzyme, significant amounts of TPN could not have been present in the mitochondria. If this reflects the true condition of the mitochondrion, it suggests that the activity of the mitochondrial TPN-linked enzyme may actually be regulated by the concentration of intramitochondrial TPN. It is also possible, however, that the mitochondrion is freely permeable to TPN, so that the pyridine nucleotide is diluted by the reaction mixture.

One difference between the results obtained here and those reported for the purified DPN-linked dehydrogenase is the relative thermal stability of the enzyme in situ. Hathaway and Atkinson (5) found that the activity of the purified enzyme is largely lost on overnight storage at $-20^\circ$. In contrast, mitochondria have been kept at $50^\circ$ for periods of up to 1 hour with no loss of DPN-linked isocitrate dehydrogenase activity.

Another difference is the second type of activation that the DPN-linked enzyme exhibits in untreated mitochondria as a result of high isocitrate concentrations or in response to heating. This apparent activation is most readily explained by the existence of a heat-labile permeability barrier which is effective in impeding the free penetration of isocitrate into the mitochondrion when the substrate level is low. Other explanations, however, are also possible, and these will be discussed in greater detail in a forthcoming communication.

This work indicates that it is possible to use a kinetic approach for the study of mitochondrial dehydrogenases in situ, although caution must be used in interpreting such kinetic measurements, because the activity of a dehydrogenase can be affected by the nature of its association with the mitochondrion as well as by the relationship between the intra- and extramitochondrial concentrations of the compounds used. Conversely, it should also be possible to use these techniques to probe the ways in which binding affects the catalytic activity of dehydrogenases.

The instrumentation, stability of the Clark electrode, and the accuracy of the oxygen determinations are judged to be very adequate, and there is no reason to suspect that the methods outlined cannot be used for more extensive kinetic analysis.

* C. Bernofsky and M. F. Utter, manuscript submitted.

The main drawback of the present system is the length of time (15 min) required for an individual determination. This, however, can be considerably shortened by the use of multiple chambers, electrodes, and recording devices.

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