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SECONDARY ACTIVATION EFFECTS OF MITOCHONDRIAL ISOCITRATE DEHYDROGENASES FROM YEAST

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SUMMARY

The kinetic properties of NAD^+-linked isocitrate dehydrogenase of yeast mitochondria have been examined by a polarographic technique which permits measurement of the enzyme *in situ*. The results not only show a cooperative interaction of the bound enzyme with isocitrate which is responsible for a substrate activation at relatively low concentrations, but also another activation which occurs at relatively high concentrations. The first substrate activation can be displaced to very low concentrations of isocitrate by the presence of AMP and was previously shown to be a property of the isolated dehydrogenase. In contrast, the second activation is not affected by AMP and has not been noted for soluble preparations. Such activation is characterized by a downward deflection of the double-reciprocal plot as the curve approaches high substrate concentrations. This non-linearity is observed only with the dehydrogenase in intact mitochondria, and it decreases or disappears when the mitochondria are heated or treated with deoxycholate. The nature of this activation has been examined further, and the data suggest that it is not the result of a direct effect on the bound dehydrogenase, but that it arises secondarily as a result of processes which increase the availability of substrate to the enzyme within the mitochondrion.

INTRODUCTION

The activation of purified yeast NAD^+-linked isocitrate dehydrogenase was first reported by KORNBERG AND PRICER[1] and later examined in greater detail by HATHAWAY AND ATKINSON[2]. The cooperative interaction of isocitrate with the enzyme gives rise to a non-classical, sigmoid, substrate *vs.* activity relationship, and in the absence of AMP the dehydrogenase shows virtually no activity at isocitrate concentrations below 0.2 mM. In the presence of AMP, however, the kinetics of this enzyme approaches that of the classical Michaelis–Menten type. The relative effect of AMP is thus greatest at low levels of isocitrate, diminishing as the concentration increases. The possible involvement of this direct type of activation in regulating the oxidation of isocitrate *in vivo via* the NAD^+-linked pathway in yeast has been suggested by BERNOFSKY

AND UTTER[3], who demonstrated that these effects hold for intact mitochondria as well as for soluble preparations of the enzyme.

During a study of the dependence on isocitrate by the NAD^+-linked dehydrogenase in intact mitochondria, it was discovered that at high levels of isocitrate, there was an additional non-classical increase in activity that was distinct from the type of activation described above. Since this could possibly represent another control mechanism for mitochondrial isocitrate oxidation, it was investigated further.

The second type of non-classical activation is characterized by a downward deflection of the double reciprocal plot as the curve approaches high substrate concentrations. This non-linearity can be diminished or abolished by conditions which either affect the integrity of the mitochondrion or cause the release of the enzyme from the particle. Experimentally, these conditions result in an increase in isocitrate dehydrogenase activity. However, unlike the action of AMP which influences only the NAD^+-linked enzyme, this activation is not specific and also affects the mitochondrial $NADP^+$-linked isocitrate dehydrogenase. This phenomenon is seen only with the bound isocitrate dehydrogenases of intact mitochondria and appears to be secondary to effects on the mitochondria themselves.

MATERIALS AND METHODS

Mitochondria

Four different preparations of yeast mitochondria were used in these studies. The mitochondria were obtained by osmotic rupture of spheroplasts as previously described[3], and they were frozen and stored at −30°. Samples which were thawed and kept at 0° are considered to be "untreated". Heated mitochondria were prepared by keeping untreated suspensions at 50° for 10 min and then chilling and maintaining them at 0°. Heating does not cause dissociation of the isocitrate dehydrogenases from the mitochondria since the enzymes can be recovered in the mitochondrial fraction isolated by centrifugation at 10 000 × *g* for 10 min.

Freezing the mitochondria does not affect the catalytic activity of the bound isocitrate dehydrogenases or their binding to the mitochondria. Although freezing predisposes certain mitochondrial functions to inactivation, intact yeast mitochondria are relatively resistant to the effects of freezing and still exhibit a high respiratory control ratio with the oxidation of α-ketoglutarate*.

Chemicals

Cis-aconitic acid was obtained from Calbiochem, Los Angeles, California. Since a polymeric product gradually forms, even in frozen solutions, this compound was dissolved and adjusted to pH 7.2 with NaOH just prior to use. The term "aconitate" in this paper refers to the *cis* isomer. Trisodium *threo*-D_8,L_8-isocitrate was the product of Sigma Chemical Company, St. Louis, Mo. The term "isocitrate" refers to the concentration of *threo*-D_8-isocitrate[4], even though the mixture of *threo* isomers was actually employed. Synthetic trisodium fluorocitrate was the kind gift of Dr. MARTIN GAL, and the concentrations reported are based on the fluorine content of the preparation.

* Unpublished data of C. BERNOFSKY AND M. F. UTTER.

NAD^+, $NADP^+$, Tris, and AMP were obtained from Sigma Chemical Co., St. Louis, Mo. Sodium deoxycholate was the product of Mann Research Laboratories Inc., New York, N.Y., and bovine serum albumin was purchased from Pentex Inc., Kankakee, Ill. Unless otherwise specified, crystalline bovine serum albumin was employed. Phenazine ethosulfate (5-ethylphenazinium ethyl sulfate) was synthesized according to McIlwain[5] and recrystallized several times from ethanol.

Other chemicals were of analytical reagent grade, and glass-distilled water was used for the preparation of all solutions. Protein was determined according to Lowry *et al.*[6] using bovine serum albumin as a standard.

Enzyme assays

Isocitrate dehydrogenase activity was determined by the method described by Bernofsky and Utter[3]. The measurement is based on the non-enzymatic oxidation of reduced pyridine nucleotides by phenazine ethosulfate, followed by the reoxidation of the reduced phenazinium salt by dissolved oxygen. Under the conditions employed, the dehydrogenation reaction is rate-limiting. Oxygen uptake was determined polarographically with a Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio). Specific activity is defined as the μmoles of O_2 taken up per min per mg of protein.

All assays contained, in addition to what is listed in the legends to the individual figures: 0.1 M Tris–chloride (pH 7.8); 6.67 mM $MgCl_2$; 5.0 mM KCN, previously neutralized with HCl; 0.2% bovine serum albumin, and 66.7 μM phenazine ethosulfate. Reactions were conducted at 30° in a final volume of 1.5 ml and were initiated by the introduction of mitochondria.

RESULTS

When the activity of the NAD^+-linked isocitrate dehydrogenase is examined as a function of isocitrate concentration and the data are presented as double reciprocal plots of activity *vs.* substrate concentration, the results summarized in Fig. 1 are obtained. In the absence of AMP (Fig. 1B), a marked non-linearity is observed over the entire concentration range tested. This has been observed by Hathaway and Atkinson[2] for the isolated enzyme and is a characteristic cooperative effect. Treatment of the mitochondria with heat or deoxycholate causes some changes in the shapes of the curves, but the general results are not altered.

In the presence of 2 mM AMP (Fig. 1A), untreated mitochondria show a linear relationship until high concentrations of isocitrate are reached. Here there is a marked activation as indicated by a downward deflection of the curve as it approaches the ordinate. Treatment with heat tends to decrease this downward deflection without altering the apparent v_{max} or the slope of the straight portion of the curve, and treatment with deoxycholate nearly abolishes this non-linearily. Although the v_{max} again appears to be unchanged in the presence of deoxycholate, the slope of the straight line portion of the curve is considerably decreased. Thus, both heat and deoxycholate treatment result in an apparent activation of the NAD^+-linked isocitrate dehydrogenase at all but the highest substrate concentrations, and to the extent that this activation is incomplete, the enzyme is further activated by substrate as indicated by the curvature near the ordinate.

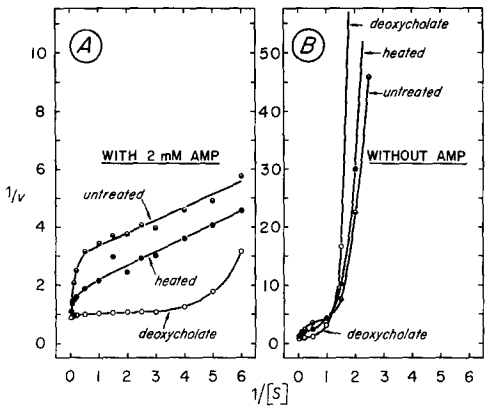


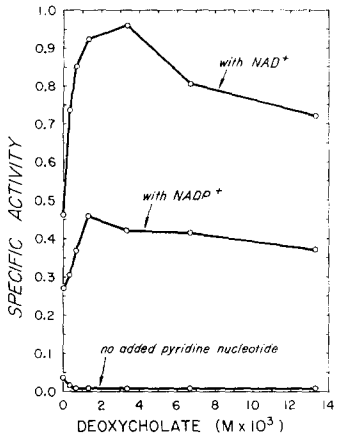
Fig. 1. Dependence of mitochondrial NAD^+-linked isocitrate dehydrogenase on isocitrate. Assays contained: 4.0 mM NAD^+, 2.0 mM AMP (when present), 2.67 mM sodium deoxycholate (when present), 0.125–0.250 mg mitochondrial protein, and isocitrate as indicated. See MATERIALS AND METHODS for other conditions and method of heating. Deoxycholate was included in the reaction medium since its effect on yeast mitochondria is instantaneous. Note that the ordinates are not the same for A and B, and that not all the experimental points are shown in B. v, specific activity; S, $10^3 \times$ molarity of isocitrate.

As can be seen in Fig. 1B, all three mitochondrial preparations exhibit a strong dependence on AMP. Judging from the relative steepness of the curves at low isocitrate concentrations, the degree of dependence seems to be related to the extent of mitochondrial disruption. It should be noted that whereas heat treatment does not lead to dissociation of the NAD^+-linked enzyme from the mitochondrion, treatment with deoxycholate completely disrupts the mitochondrial structure, and the dehydrogenase is found in the supernatant fraction following centrifugation at 100 000 $\times$ g for 100 min.

Although it is evident that treatment with heat and deoxycholate leads to substantial increases in the apparent activity of NAD^+-linked isocitrate dehydrogenase, it is important to know whether such changes are specific for this enzyme. If they are, it would suggest that the activating conditions are directly affecting the NAD^+-linked dehydrogenase in a way which is linked to a control mechanism for this enzyme. On the other hand, if these effects are not specific, it is likely that the activating conditions are operating in an indirect and more general manner. Accordingly, mitochondria were treated with deoxycholate, and both the $NADP^+$ and NAD^+-linked isocitrate dehydrogenases were assayed. The $NADP^+$-linked enzyme provides a very convenient system for testing this question, since it utilizes the same substrate.

As seen in Fig. 2, both enzymes are activated by the bile salt. This indicates that the activation is not specific with regard to the dehydrogenase, and that it most likely involves isocitrate and its ability to act as a substrate for the enzymes *in situ*.

Since secondary activation can be produced by damage or disruption of the mitochondrion, the question arises as to whether the activation at high isocitrate concentrations is also the result of mitochondrial damage. To test this, mitochondria were preincubated with 20 mM isocitrate under the usual assay conditions. The mitochondria were then reisolated by centrifugation, resuspended, and then examined for secondary activation. Fig. 3 shows that the reisolated mitochondria exhibit a



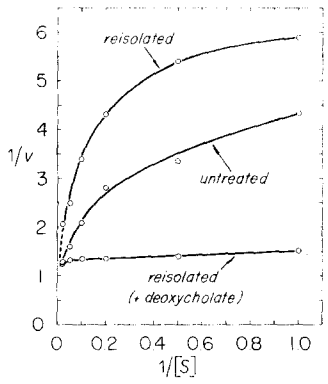


Fig. 2. Effect of deoxycholate on mitochondrial NAD^+- and $NADP^+$-linked isocitrate dehydrogenases. Assays contained: 2.67 mM AMP, 5.0 mM isocitrate, 5.33 mM NAD^+ (when present), 2.67 mM $NADP^+$ (when present), 0.250 mg mitochondrial protein, and sodium deoxycholate as indicated. See MATERIALS AND METHODS for other conditions. In the absence of deoxycholate, the activity with no added pyridine nucleotide at 5 mM isocitrate is only 15% of what it is at 50 mM isocitrate[8], suggesting that the higher substrate concentration retards the release of bound NAD^+ from the mitochondria.

Fig. 3. Effect of pretreatment on the isocitrate dependence of mitochondrial NAD^+-linked isocitrate dehydrogenase. Assays contained: 4.0 mM NAD^+, 2.5 mM AMP, 2.0 mM sodium deoxycholate (when present), 0.197 mg mitochondrial protein, and isocitrate as indicated. See MATERIALS AND METHODS for other conditions. Reisolated mitochondria were prepared by preincubating untreated mitochondria at a final protein concentration of 0.985 mg/ml for 5 min at 30° in the above assay medium with 20 mM isocitrate. The mitochondria were chilled with ice, centrifuged at 10 000 × *g* for 15 min, resuspended in the cold with a small volume of 20% (w/v) sucrose, 1 mM disodium EDTA and 50 mM potassium phosphate (pH 6.8), and then assayed as above. v, specific activity; S, 10^3 × molarity of isocitrate.

greater degree of substrate activation at high concentrations of isocitrate, although the v_{max} appears to be unchanged. These data indicate that whatever factor is responsible for the lack of activity in untreated mitochondria has been enhanced by the preincubation treatment. The substrate activation thus seems to be reversible and does not involve mitochondrial disruption.

Fig. 3 shows further that when the reisolated mitochondria are disrupted with deoxycholate, there is obtained a full expression of isocitrate dehydrogenase activity. These data suggest that secondary activation is not a fixed property of the mitochondrion, but rather one which is dependent on the prior conditions to which the mitochondrion has been subjected.

The activation which results from high substrate, heat, and deoxycholate can be explained readily in terms of mitochondrial permeability. That is, the apparent activation phenomenon may be due to a mitochondrial barrier against isocitrate which can be overcome by high concentrations of substrate, or altered or destroyed by heat or deoxycholate. However, other explanations are also possible. For instance, the activating conditions might affect a mitochondrial inhibitor, or somehow alter the active sites of the enzymes themselves. It is also possible that there is competition by other pathways for the intramitochondrial isocitrate available for oxidation. Activation would then result either from saturation of these pathways with substrate, or from destruction of these pathways with heat or deoxycholate. In the present

study, this last alternative was examined as a possible mechanism for the observed secondary activating effects.

The reactions competing with isocitrate oxidation in yeast are the conversion of isocitrate to citrate by aconitase and the cleavage of isocitrate to succinate and glyoxylate by isocitratase. Since the position of equilibrium of the aconitase reaction strongly favors citrate formation[7], it was thought that the aconitase pathway would be the most likely competitor, and that the activation of isocitrate dehydrogenase could be a reflection of aconitase inhibition or inactivation. An attempt was therefore made to characterize the interaction of aconitase with the mitochondrial isocitrate dehydrogenase system.

In Fig. 4 the activity of the mitochondrial NAD^+-linked enzyme, presented in double reciprocal form, is shown as a function of either isocitrate or aconitate concen-

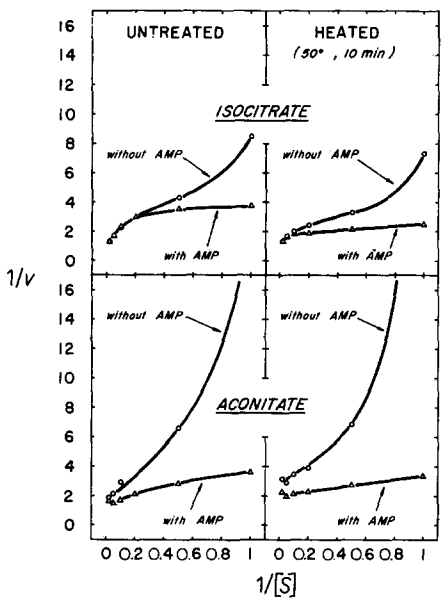


Fig. 4. Dependence of mitochondrial NAD^+-linked isocitrate dehydrogenase on isocitrate and aconitate. Assays contained: 2.0 mM NAD^+, 2.67 mM AMP (when present), 0.232 mg mitochondrial protein, and isocitrate and aconitate as indicated. Fraction V bovine serum albumin was used instead of the crystalline product. See MATERIALS AND METHODS for other conditions. Note that not all the experimental points are shown with aconitate. v, specific activity; S, $10^3 \times$ molarity of substrate.

tration. With untreated mitochondria, substitution of aconitate for isocitrate in the presence of AMP is seen to result in an overall activation at relatively high concentrations of substrate, and there is little further activation near v_{max}. In addition, the effect of heating on the reaction with aconitate as the substrate is considerably smaller than it is with isocitrate.

Paradoxically, aconitate appears to be a better source of intramitochondrial isocitrate than is isocitrate itself, although this is true only for the substrate range of 1 to 20 mM. Fig. 5 shows that with untreated mitochondria, the activity curves obtained with aconitate and isocitrate cross at two points, one at 20 mM (aconitate is

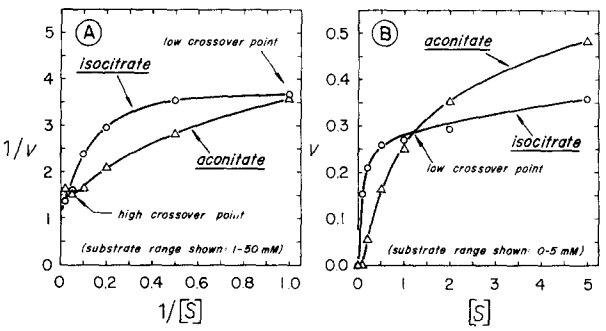


Fig. 5. Dependence of mitochondrial NAD+-linked isocitrate dehydrogenase on isocitrate and aconitate. The data in A are those of Fig. 4 in the presence of AMP. The assays for B contained: 3.33 mM NAD^+, 2.5 mM AMP, 0.197 mg mitochondrial protein, and isocitrate and aconitate as indicated. See MATERIALS AND METHODS for other conditions. Note that A is a double reciprocal plot covering the high range of substrate concentrations, while B is a plot of velocity *vs.* substrate concentration covering the low range of concentrations. A and B represent two separate experiments. *v*, specific activity; *S*, $10^3 \times$ molarity of substrate.

inhibitory at very high concentrations), and the other at 1 mM. At concentrations lower than 1 mM, the rate of conversion of aconitate to isocitrate is limiting to the extent that aconitate is no longer a better source of intramitochondrial isocitrate than is isocitrate. The higher activity with aconitate in the 1–20 mM range is probably the result of the relatively unimpeded penetration of aconitate into the mitochondrion combined with the high specific activity of mitochondrial aconitase*.

Although these findings strengthen the possible role of permeability factors in secondary activation, the presence of active aconitase makes it necessary to evaluate the importance of this enzyme in the activation process.

In the studies which follow, the assays of aconitase are based on the activities of mitochondrial NAD^+-linked isocitrate dehydrogenase when aconitate is the initial

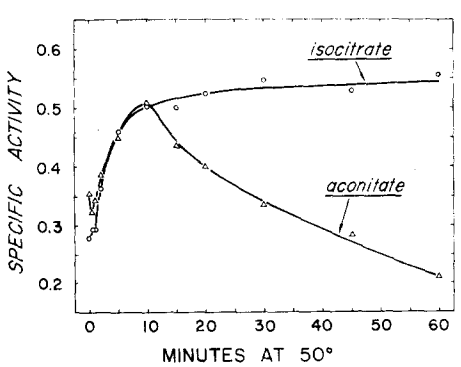


Fig. 6. Effect of heat on mitochondrial NAD^+-linked isocitrate dehydrogenase and aconitase. Assays contained: 2.0 mM NAD^+, 2.67 mM AMP, 2.0 mM isocitrate or aconitate as indicated, and 0.232 mg mitochondrial protein. For each determination, a mitochondrial suspension was maintained at 50° for a given length of time, then chilled with ice and assayed at 30°. See MATERIALS AND METHODS for other conditions.

* The specific activity of aconitase in yeast mitochondria is more than twice that of NAD^+-linked isocitrate dehydrogenase (C. BERNOFSKY AND M. F. UTTER, unpublished observation).

substrate. Since the dehydrogenase is rate-limiting in untreated mitochondria, a true measure of aconitase is not obtained until its activity falls below that of the dehydrogenase. Nevertheless, it is still possible to use this system to examine aconitase during its inactivation, since the activity of this enzyme will eventually become rate-limiting, assuming that there is no corresponding decrease in the activity of the NAD^+-linked isocitrate dehydrogenase.

In order to test the possibility that the activation of isocitrate dehydrogenase is due to the destruction of aconitase and the consequent removal of a competing pathway, the effects of heat, deoxycholate and fluorocitrate on the activities of aconitase and NAD^+-linked isocitrate dehydrogenase were investigated.

To test the effects of heat, a mitochondrial suspension was maintained at 50° for various lengths of time and then chilled with ice and assayed with 2 mM isocitrate or aconitate. As seen in Fig. 6, the initial samples are more active with aconitate than with isocitrate, in accord with the data in Fig. 5 for this substrate concentration. Upon heating, the activity of the mitochondrial NAD^+-linked isocitrate dehydrogenase increases steadily up to 10 min, and between 2 and 10 min there is little difference in the relative activities with the two substrates. Upon longer heat treatment, however, there is a steady decline of activity when aconitate is the initial substrate, but there is no change in the activity with isocitrate. This shows that aconitase, but not isocitrate dehydrogenase is inactivated by the heating.

Although heating inactivates aconitase, this is not reflected during the first 10 min because aconitase has not yet become rate-limiting. The increase in the activity with both aconitate and isocitrate during this period is probably the result of an enhancement in mitochondrial permeability to these substrates. While aconitate is relatively more permeable than isocitrate, its penetration, particularly at lower concentrations is still somewhat restricted, and at 2 mM aconitate, heating for 10 min causes a resultant increase in activity. When permeability restrictions are initially overcome, such as at high aconitate concentrations, then the destruction of activity upon heating becomes a more prominent factor. Because of these opposing effects, heating for 10 min can lead to either an increase or a decrease of aconitase activity, depending on the aconitate concentration, and the double reciprocal plots in Fig. 4 of heated and untreated mitochondria cross each other at a concentration of 3 mM.

In contrast, heating under the conditions employed does not destroy isocitrate dehydrogenase, and the activity with isocitrate increases at all but the highest substrate concentrations (Figs. 1A and 4). The ability of the *in situ* NAD^+-linked isocitrate dehydrogenase to withstand a temperature of 50° for 1 h with no loss of activity is noteworthy for an enzyme which is quite unstable in its isolated state[2]. The association of the dehydrogenase with the mitochondrion evidently confers upon it a strong resistance to denaturation.

The above experiments fail to establish a relationship between the heat activation of NAD^+-linked isocitrate dehydrogenase and the destruction of aconitase. However, since it was known that a greater degree of activation could be produced by the action of deoxycholate, the effect of this agent on the NAD^+-linked dehydrogenase was also investigated. Using 5 mM isocitrate, aconitate, or citrate as the initial substrates an attempt was made to see if there was any demonstrable relationship between aconitase activity and the activation of isocitrate dehydrogenase.

Fig. 7 shows that at concentrations of deoxycholate less than 0.1 mM, where the

bile salt has little effect, isocitrate dehydrogenase activity is greater with aconitate than with isocitrate as the substrate. As the level of deoxycholate approaches the optimum "activating" concentration of 1.8 mM, there is an increase in isocitrate dehydrogenase activity. However, this increase is not associated with a visible decrease in aconitase activity, although a decrease in aconitase would not be detected unless its activity dropped to rate-limiting values. Fig. 7 actually shows a rise in aconitase activity. This is probably a reflection of an increase in the access of aconitate to aconitase as the mitochondria are disrupted by the action of deoxycholate. The increase with isocitrate appears to be analogous, although it is greater in extent because the undamaged mitochondria are initially less permeable to isocitrate than to aconitate.

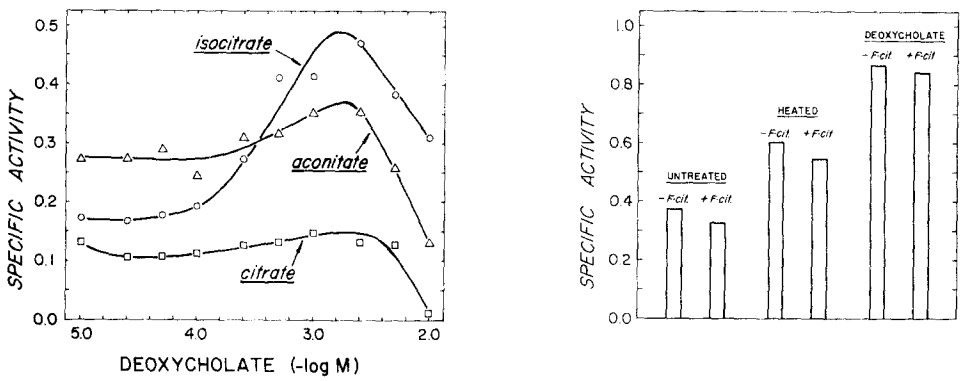


Fig. 7. Effect of deoxycholate on mitochondrial NAD+-linked isocitrate dehydrogenase and aconitase. Assays contained: 2.0 mM NAD^+; 2.67 mM AMP; 5.0 mM citrate, aconitate, or isocitrate; 0.300 mg mitochondrial protein, and sodium deoxycholate as indicated. See MATERIALS AND METHODS for other conditions.

Fig. 8. Effect of fluorocitrate on mitochondrial NAD+-linked isocitrate dehydrogenase. Assays contained: 2.0 mM NAD^+, 5.0 mM isocitrate, 11.6 mM fluorocitrate (when present), 0.150 mg mitochondrial protein, and 1.78 mM sodium deoxycholate (when present). See MATERIALS AND METHODS for other conditions and method of heating.

It is of interest that citrate is only poorly utilized over the entire range of conditions from intact mitochondria to mitochondria completely disrupted by deoxycholate.

As seen in Fig. 7, high levels of deoxycholate inactivate isocitrate dehydrogenase. It was determined in a separate experiment that both aconitase and the NAD^+-linked isocitrate dehydrogenase of yeast mitochondria are inhibited approx. 50% by 20 mM deoxycholate. These measurements were made with 20 mM substrate, and aconitase was assayed in the presence of $NADP^+$ and excess $NADP^+$-linked isocitrate dehydrogenase.

The study with deoxycholate also fails to indicate any relationship between the activation of mitochondrial NAD^+-linked isocitrate dehydrogenase and an inactivation of aconitase. However, one further attempt was made to implicate aconitase in this process by using fluorocitrate, a specific inhibitor of aconitase.

The effect of 11.6 mM fluorocitrate on NAD^+-linked isocitrate dehydrogenase is shown in Fig. 8. It can be seen that with untreated mitochondria, the presence of the inhibitor does not lead to the activation of isocitrate dehydrogenase. In addition,

treatment of aconitase with fluorocitrate in the heat and deoxycholate-activated preparations does not further enhance the activity of isocitrate dehydrogenase.

Because of the apparent lack of a fluorocitrate effect, the action of this inhibitor on mitochondrial aconitase was investigated further. Using 5 mM citrate or aconitate as substrate, aconitase was assayed by measuring the activity of mitochondrial NAD^+-linked isocitrate dehydrogenase. From the results presented in Fig. 9, it can

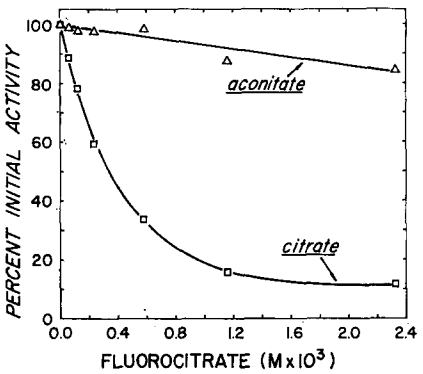


Fig. 9. Inhibition of mitochondrial aconitase by fluorocitrate. Assays contained: 2.0 mM NAD^+, 2.67 mM AMP, 0.150–0.300 mg mitochondrial protein, 5.0 mM citrate or aconitate, and fluorocitrate as indicated. See MATERIALS AND METHODS for other conditions. Initial specific activities are 0.279 with citrate and 0.588 with aconitate.

be seen that the formation of isocitrate from citrate is inhibited by fluorocitrate to a much greater extent than is the formation of isocitrate from aconitate. Unfortunately this experiment with fluorocitrate is not conclusive because of the incomplete inhibition of aconitase.

The above attempts to implicate aconitase in the secondary activation process are based on the premise that a rapid conversion of isocitrate to citrate could effectively compete with isocitrate oxidation for available substrate. However, no evidence has been obtained to indicate that destruction of the aconitase pathway is responsible for the activation of isocitrate dehydrogenase.

DISCUSSION

The present experiments indicate that aconitase does not mediate a pathway that is significantly competitive with isocitrate oxidation in yeast mitochondria. Judging from the high activity of mitochondrial aconitase, this finding is somewhat surprising since it would be expected that isocitrate is rapidly converted to citrate. That this is not so may simply reflect the relative rates of the various aconitase reactions. MORRISON[8] reported that the conversion of aconitate to isocitrate by purified aconitase from pig heart was nearly twice the rate of the conversion of isocitrate to citrate. An alternative explanation is that *in situ* yeast aconitase is subject to some type of control which differentially affects its activities. Finally, it is possible that because the polarographic method measures the initial rates of the oxidative pathway, the influence of other pathways is minimized. The effect of alternate pathways on the

oxidation of isocitrate in untreated mitochondria cannot be ruled out entirely, however, until more is known about the relative rates of isocitrate utilization by all of the pathways present.

When mitochondria are heated, there is an increase in isocitrate dehydrogenase activity at all but the highest concentrations of isocitrate. This phenomenon is probably related to the reproducible activation reported by CHEN AND PLAUT[9] when a crude NAD^+-linked isocitrate dehydrogenase preparation from bovine heart, assayed at 2.67 mM isocitrate, is subjected to heating at 50° for 15 min. HATHAWAY AND ATKINSON[2] have also reported increases in total activity during the first stages of purification of the enzyme from yeast. Thus, reliable values for the activity of this enzyme, particularly in its unisolated state, can only be obtained by measuring the v_{max} per mg protein. Alternatively, sodium deoxycholate can be used to simplify the assay of the crude enzyme.

One means of explaining the data with untreated and heated mitochondria (Fig. 1A), is to postulate the existence of a mitochondrial inhibitor. WIGGERT AND COHEN[10] have shown that L-alanine, an inhibitor of frog-liver glutamate dehydrogenase, produces a type of inhibition that is apparently competitive at high concentrations of glutamate, and uncompetitive at lower concentrations of glutamate. The double reciprocal plots bear a close resemblance to the plots obtained here with untreated and heated mitochondria in the presence of AMP. In addition, WRATTEN AND CLELAND[11] have presented data which show that methanol inhibits horse-liver alcohol dehydrogenase in a similar manner. Thus, the activation produced by heating could be explained by the existence of a heat-labile inhibitor of both the $NADP^+$- and NAD^+-linked isocitrate dehydrogenases which is destroyed or diluted by deoxycholate treatment and counteracted by aconitate. Although possible, this combination of circumstances seems unlikely.

A simple and consistent explanation for the various activation phenomena presented here can be formulated on the assumption that the dehydrogenase within the intact mitochondrion is subject to certain physical restrictions which prevent it from interacting with its substrate. The first such restriction would be imposed by the semi-permeable membrane which prevents the equilibration of substrate between the intra- and extramitochondrial compartments. The second type of constraint would be caused by attachment of the dehydrogenase to a structural component within the mitochondrion. This would restrict the translational freedom of the enzyme and hence its interaction with substrate molecules within the intramitochondrial compartment. These two factors could cause the bound enzyme to exist in an apparently inhibited state in the intact mitochondrion, and both factors may be involved in the present studies. Treatment of the mitochondrion with deoxycholate leads to the situation where the dehydrogenase is simply freed of all mitochondrial restraints. The other types of activation, however, are more complex.

The non-classical increase in activity at high substrate concentrations probably arises when the concentration of substrate exceeds the capacity of the semi-permeable membrane to maintain a concentration gradient between the intra- and extramitochondrial compartments. In this context, the relative absence of a strong permeability barrier against aconitate is consistent with the much smaller non-classical increase seen at high concentrations of this substrate (see Figs. 4 and 5). The effect of heat can also be explained in terms of damage to the mitochondrial permeability barrier and

the consequent equilibration of substrate between the two compartments. Moreover, to the extent that the semi-permeable membrane is not damaged by heat, further substrate activation can occur (see Fig. 1A).

Heating alone does not produce the same degree of activation obtained with deoxycholate (see Fig. 1A), and extensive heating only leads to destruction of the enzyme and a decrease of the v_{max} per mg protein. It should be emphasized that after heating the mitochondrial structure is still relatively intact, and there is no release of the dehydrogenase from the particle. In this situation the integrity of the semi-permeable membrane has possibly been destroyed; however, the second constraint due to binding of the enzyme to the mitochondrial structure still exists. It is apparent that maximal expression of dehydrogenase activity is possible only when the enzyme has been freed of all structural associations.

If these views are correct, it means that the imposition of structural restraints upon the interaction of an enzyme with its substrate can produce a kinetic outcome indistinguishable from that of inhibition. In such cases, no change in the v_{max} is expected since the restrictions influence only the effective concentration of substrate. A somewhat similar situation has been observed by DALLAM AND REED[12] who showed that when rat-liver mitochondria swell in response to thyroxin, there is an associated increase in the oxidation of ferrocytochrome *c* and NADH.

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