The Oxidation of Tricarboxylate Anions by Mitochondria Isolated from *Neurospora crassa*

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(Received 14 November 1980; revised 10 February 1981)

Mitochondria isolated from *Neurospora crassa* grown with sucrose as the sole source of carbon and energy oxidized citrate and *cis*-aconitate at high rates, and showed good respiratory control and high ADP/O ratios. The oxidation of both substrates was inhibited strongly by rotenone but only slightly by malonate. In contrast, isocitrate was oxidized slowly, with poor respiratory control. Treatment with detergent showed that both NAD⁺- and NADP⁺-isocitrate dehydrogenases were situated within the mitochondrial matrix. It thus appeared that the rate of oxidation of exogenous isocitrate was limited by a slow rate of translocation across the inner mitochondrial membrane.

When *N. crassa* was grown in an acetate-containing medium, the mitochondrial pellet, prepared by differential centrifugation, catalysed a rapid oxidation of isocitrate and contained a high activity of isocitrate lyase. The oxidation of isocitrate and succinate was strongly inhibited by malonate but only slightly by rotenone. Density-gradient centrifugation revealed that the apparent oxidation of isocitrate by mitochondrial pellets was due to contamination by glyoxysomes. Isocitrate was converted into glyoxylate and succinate in the glyoxysomes, then succinate was translocated across the inner mitochondrial membrane and oxidized by the respiratory chain.

INTRODUCTION

Previous studies have shown that mitochondria isolated from *Neurospora crassa* oxidize citrate with a P/O ratio of between 1.8 and 3.0 (Hall & Greenawalt, 1967; Lambowitz *et al.*, 1972). There is little information concerning the oxidation of *cis*-aconitate and isocitrate in *N. crassa* mitochondria. All three substrates are ultimately oxidized by isocitrate dehydrogenase, but the rate of oxidation of citrate and *cis*-aconitate may be limited by the activity of aconitate hydratase. The rate of translocation of tricarboxylate anions across the inner membrane of the mitochondria could also be a factor limiting the rates of oxidation of these substrates. The existence of translocators for phosphate and dicarboxylate anions was shown by Katkocin & Slayman (1976). There is, however, no information available concerning the translocation of tricarboxylate anions across the inner membrane of *N. crassa* mitochondria.

A major purpose of this work was to investigate the ability of mitochondria isolated from *N. crassa* to oxidize citrate, *cis*-aconitate and isocitrate after the hyphae had been grown in the presence of sucrose or acetate for different periods of time. The oxidation of isocitrate produced by the mitochondria is compared with the oxidation of external isocitrate. The results are discussed on the basis of the activity and the localization of enzymes catalysing isocitrate breakdown, emphasizing the possible interactions between mitochondria and glyoxysomes, which contain a large amount of isocitrate lyase when *N. crassa* is grown in acetate-containing medium (Kobr *et al.*, 1969).

METHODS

The growth conditions to obtain hyphae of *Neurospora crassa* (wild type, strain STA4) and the procedures used to isolate and purify the mitochondria, measure the enzyme activities and assay protein have been described in the accompanying paper (Schwitzguébel *et al.*, 1981).

Measurement of respiratory activity. The rate of oxygen consumption was measured polarographically using a Clark-type oxygen electrode. Each assay was carried out at 25 °C, with 0.2 to 0.5 mg of mitochondrial protein in a final volume of 1 ml of the assay medium (0.3 M-sucrose, 10 mM-potassium phosphate buffer, pH 7.0, 10 mM-KCl, 5 mM-MgCl₂, 1 mM-EGTA, 0.1% bovine serum albumin). Assays were done with 20 mM-citrate (BDH), 20 mM-*cis*-aconitate (Calbiochem) and 40 mM-DL-isocitrate (Sigma) as substrates, in the presence of 1 mM-L-malate (Sigma). All the substrates were measured gravimetrically, dissolved to give concentrated solutions in distilled water, neutralized and stored frozen. When needed, quantitative additions of ADP (Boehringer) were made (final concentration 0.1 to 0.5 mM). Malonate (Sigma) at a final concentration of 2.5 mM was used as an inhibitor of succinate dehydrogenase. Rotenone (Sigma) at a final concentration of 20 µM was used as an inhibitor of the internal NADH hydrogenase. It was freshly prepared as a solution in dimethyl sulphoxide. ADP/O ratios were calculated from the oxygen electrode traces after quantitative additions of ADP.

RESULTS

N. crassa grown with sucrose

Mitochondria isolated from *N. crassa* harvested in the late exponential phase of growth (24 h) were able to oxidize citrate and *cis*-aconitate at high rates (Table 1*a*). The respiratory control values ranged between 3.2 and 3.6 and the ADP/O ratios were about 2.5. The oxidation of both substrates was strongly inhibited by rotenone, but only slightly inhibited by malonate. The rate of oxygen consumption in the presence of 40 mM-DL-isocitrate was 20% of that obtained when citrate or *cis*-aconitate was supplied as the substrate. The oxidation of isocitrate was not inhibited by malonate and only 50% reduced by rotenone (Table 1*a*). This apparent resistance to rotenone may be due to the rather slow uninhibited rate. The rate of isocitrate oxidation could not be enhanced by increasing the concentration of DL-isocitrate to 100 mM, by changing the pH of the assay medium between 6.7 and 7.7 or by adding 0.5 mM-NAD⁺, 0.5 mM-NADP⁺ or 0.25 mM-AMP. Furthermore, no clear transition between state 3 and state 4 was observed, and further additions of ADP failed to increase the rate of oxidation of isocitrate.

The maximal activities of NAD⁺- and NADP⁺-isocitrate dehydrogenases, assayed in the presence of Triton X-100 (Table 2*a*), were in the same order of magnitude as the rate of oxidation of citrate and *cis*-aconitate by intact mitochondria (Table 1*a*). The activity of these enzymes could represent the rate-limiting step in the oxidation of both citrate and *cis*-aconitate in mitochondria from hyphae grown for 24 h. Approximately 96% of both NAD⁺- and NADP⁺-isocitrate dehydrogenase activities were located inside the inner mitochondrial membrane, as indicated by the 20-fold increase in the ability of isocitrate to reduce NAD⁺ or NADP⁺ following the addition of Triton X-100 to the mitochondria. The remaining 4% of the enzyme activities found in the supernatant was probably released from the broken mitochondria, and was capable only of reducing 13 nmol NAD(P)⁺ min⁻¹ (mg protein)⁻¹. This rate was considerably lower than that necessary to support isocitrate oxidation, which occurred at a rate equivalent to 72 nmol min⁻¹ (mg protein)⁻¹. It is therefore postulated that isocitrate must be transported into the mitochondrial matrix before oxidation can take place, and a slow rate of translocation could limit the rate of oxidation.

When *N. crassa* was harvested in the stage at which differentiation into conidia occurred (96 h), isolated mitochondria contained amounts of NAD⁺- and NADP⁺-isocitrate dehydrogenases similar to those found in mitochondria from hyphae from 24 h cultures (Table 2*a*). In contrast, the rates of oxidation of citrate and *cis*-aconitate were 50% lower than those observed in mitochondria from younger cultures (compare Tables 1*a* and 1*b*). In addition, the oxidation of both substrates was less sensitive to inhibition by rotenone and was less efficiently coupled to ATP synthesis, as reflected in lower ADP/O ratios. Mitochondria

Table 1. Oxidation of tricarboxylate anions by intact mitochondria from *N. crassa* grown with sucrose

The uptake of oxygen [$\text{nmol O}_2 \text{ min}^{-1} (\text{mg protein})^{-1}$] is expressed as mean values $\pm$ standard errors. The number of determinations is given in parentheses. Concentrations are indicated in Methods.

(a) Cultures grown for 24 h

	Citrate	<i>cis</i> -Aconitate	Isocitrate
State 3 oxidation	171 $\pm$ 6 (5)	170 $\pm$ 8 (5)	36 $\pm$ 3 (9)
+ Rotenone	26 $\pm$ 2 (5)	24 $\pm$ 2 (3)	18 $\pm$ 2 (4)
+ Malonate	147 $\pm$ 9 (3)	145 $\pm$ 7 (3)	35 $\pm$ 2 (3)

(b) Cultures grown for 96 h

	Citrate	<i>cis</i> -Aconitate	Isocitrate
State 3 oxidation	84 $\pm$ 5 (6)	88 $\pm$ 8 (4)	40 $\pm$ 3 (4)
+ Rotenone	20 $\pm$ 2 (6)	21 $\pm$ 1 (4)	33 $\pm$ 2 (2)
+ Malonate	76 $\pm$ 4 (3)	83 $\pm$ 1 (3)	14 $\pm$ 1 (4)

Table 2. Activity of particulate enzymes catalysing isocitrate breakdown in extracts from *N. crassa*

The enzyme activities [$\text{nmol isocitrate consumed min}^{-1} (\text{mg protein})^{-1}$] are expressed as mean values $\pm$ standard errors. The number of determinations is given in parentheses. Enzyme activities were measured in the presence of 0.02% (w/v) Triton X-100.

(a) Cultures grown with sucrose

	NAD ⁺ -isocitrate dehydrogenase	NADP ⁺ -isocitrate dehydrogenase	Isocitrate lyase
24 h cultures	260 $\pm$ 21 (4)	73 $\pm$ 18 (4)	14 $\pm$ 5 (4)
96 h cultures	273 $\pm$ 43 (3)	59 $\pm$ 4 (3)	59 $\pm$ 25 (3)

(b) Cultures grown with acetate

	NAD ⁺ -isocitrate dehydrogenase	NADP ⁺ -isocitrate dehydrogenase	Isocitrate lyase
48 h cultures	392 $\pm$ 50 (5)	210 $\pm$ 50 (5)	192 $\pm$ 95 (4)
66 h cultures (washed mitochondria)	250 $\pm$ 73 (3)	142 $\pm$ 44 (3)	130 $\pm$ 45 (3)
66 h cultures (mitochondria purified in a Percoll gradient)	437 $\pm$ 71 (2)	191 $\pm$ 25 (2)	9 (1)

isolated from 96 h cultures thus appeared to have a modified NADH dehydrogenase linked to the respiratory chain. The rate of oxidation of isocitrate in mitochondria from 96 h cultures was slow, relatively resistant to rotenone and sensitive to inhibition by malonate (Table 1 b). It had, therefore, some of the characteristics of the oxidation of succinate. Furthermore, the activity of isocitrate lyase, which cleaves isocitrate into glyoxylate and succinate, was relatively high in this preparation of mitochondria (Table 2 a). Nabeshima *et al.* (1977) reported that the isocitrate lyase activity of *Candida tropicalis* grown with sucrose increased after the middle phase of growth. The results presented here indicate that such a situation exists also in *N. crassa*. This is in agreement with previous publications showing that acetate accumulates within the cells as the culture ages (Zink, 1967) and the synthesis of isocitrate lyase is derepressed when *N. crassa* is grown with acetate (Kobr *et al.*, 1975).

Table 3. *Oxidation of tricarboxylate anions by intact mitochondria from N. crassa grown with acetate*

The uptake of oxygen [$\text{nmol O}_2 \text{ min}^{-1} (\text{mg protein})^{-1}$] is expressed as mean values $\pm$ standard errors. The number of determinations is given in parentheses. Concentrations are indicated in Methods.

(a) *Cultures grown for 48 h*

	Citrate	<i>cis</i> -Aconitate	Isocitrate
State 3 oxidation	176 $\pm$ 9 (7)	161 $\pm$ 8 (4)	87 $\pm$ 6 (7)
+ Rotenone	26 $\pm$ 1 (5)	20 $\pm$ 2 (6)	58 $\pm$ 7 (5)
+ Malonate	150 $\pm$ 14 (2)	135 $\pm$ 3 (3)	21 $\pm$ 2 (6)

(b) *Cultures grown for 66 h (washed mitochondria)*

	Citrate	<i>cis</i> -Aconitate	Isocitrate
State 3 oxidation	53 $\pm$ 3 (5)	48 $\pm$ 4 (6)	50 $\pm$ 5 (6)
+ Rotenone	15 $\pm$ 2 (4)	17 $\pm$ 2 (3)	43 $\pm$ 7 (3)
+ Malonate	49 $\pm$ 5 (5)	46 $\pm$ 5 (2)	22 $\pm$ 3 (4)

(c) *Cultures grown for 66 h (mitochondria purified in a Percoll gradient)*

	Citrate	<i>cis</i> -Aconitate	Isocitrate
State 3 oxidation	46 $\pm$ 4 (4)	52 $\pm$ 4 (3)	17 $\pm$ 1 (3)
+ Rotenone	15 $\pm$ 2 (2)	22 (1)	12 $\pm$ 2 (2)
+ Malonate	39 (1)	47 (1)	14 $\pm$ 2 (2)

N. crassa grown with acetate

When *N. crassa* was harvested in the late exponential phase of growth (48 h), the rates of oxidation of isocitrate and *cis*-aconitate by isolated mitochondria, the respiratory control values and the ADP/O ratios were similar to those obtained with mitochondria prepared from hyphae grown for 24 h with sucrose (compare Tables 1*a* and 3*a*). These mitochondria were found to contain 50% more NAD^+ -isocitrate dehydrogenase and three times as much NADP^+ -isocitrate dehydrogenase as present in mitochondria obtained from hyphae grown with sucrose (Table 2*a, b*). Thus the activity of the two isocitrate dehydrogenases does not appear to be a rate-limiting step in the oxidation of citrate or *cis*-aconitate in mitochondria prepared from cultures grown in the presence of acetate, in contrast to the situation described for *N. crassa* grown with sucrose.

The rate of oxidation of isocitrate by mitochondrial pellets obtained from hyphae grown in the presence of acetate was two to three times greater than the rate measured under sucrose-grown conditions (compare Tables 1*a* and 3*a*) and reached 50% of that obtained for the oxidation of other tricarboxylate anions. The oxidation of citrate and *cis*-aconitate attained a maximal and constant rate 1 min after adding the substrate, whereas the maximal rate of isocitrate oxidation was not reached until 6 min after adding the substrate. The oxidation of isocitrate also differed from the oxidation of citrate and *cis*-aconitate in that it was relatively unaffected by rotenone and strongly inhibited by malonate (Table 3*a*). A low sensitivity to rotenone and a strong inhibition by malonate are also characteristic of the oxidation of succinate.

The activity of isocitrate lyase in the mitochondrial pellet was greatly enhanced when *N. crassa* was grown in the presence of acetate (Table 2*b*). In contrast to the activity of the two isocitrate dehydrogenases, that of isocitrate lyase was not increased upon the addition of Triton X-100 to the intact mitochondria. This therefore suggests that isocitrate lyase cleaved isocitrate into glyoxylate and succinate outside the matrix, succinate then being translocated across the inner mitochondrial membrane and oxidized in a malonate-sensitive and

Fig. 1. Effects of glyoxysomes on the oxidation of isocitrate by purified mitochondria. Mitochondria and glyoxysomes were isolated from *N. crassa* grown for 66 h on acetate, and were purified on a Percoll gradient. Respiration rates (indicated on the traces) are expressed as nmol O₂ consumed min⁻¹. The reaction mixture contained 40 mM-DL-isocitrate (+ 1 mM-L-malate), 2.5 mM-malonate, 0.5 mM-ADP, 0.43 mg mitochondrial protein and 0.35 mg glyoxysomal protein. The specific activity of isocitrate lyase [nmol glyoxylate produced min⁻¹ (mg protein)⁻¹] was 9 in the mitochondria and 72 in the glyoxysomes.

rotenone-resistant manner. The time required to produce a sufficient amount of succinate could explain the long period of time needed to reach the maximal linear rate of oxygen uptake in the presence of isocitrate.

When *N. crassa* was harvested in the stationary phase of growth (66 h), the rates of oxidation of citrate and *cis*-aconitate by mitochondrial pellets represented only one-third of the rates observed with mitochondria from hyphae still growing exponentially (Table 3*b*). This reduction in the rates of oxidation as the culture aged was accompanied by a reduction in the ADP/O ratio from 2.5 to 1.5, a decrease of the respiratory control from 4 to 2 and a decrease in sensitivity to rotenone. This suggests that some modification of the respiratory chain occurred, possibly at the level of the NADH dehydrogenase and the first energy-coupling site. The activities of both NAD⁺- and NADP⁺-isocitrate dehydrogenases were somewhat reduced, but were still more than sufficient to mediate the oxidation of tricarboxylate anions (Table 2*b*). Although the rate of oxidation of isocitrate was quantitatively similar to that of the other tricarboxylate anions, it differed qualitatively in being resistant to rotenone and sensitive to malonate (Table 3*b*). It thus had many of the characteristics associated with the oxidation of succinate. Since isocitrate lyase activity was high in the mitochondrial pellet, the apparent oxidation of isocitrate could be the result of a contamination by glyoxysomes, which are known to contain isocitrate lyase when *N. crassa* is grown with acetate (Kobr *et al.*, 1969).

When mitochondria were separated from glyoxysomes by density-gradient centrifugation on Percoll, the rates of oxidation of citrate and *cis*-aconitate were not increased (Table 3*c*), although a slight enrichment in NAD⁺- and NADP⁺-isocitrate dehydrogenases was observed (Table 2*b*). In contrast, the oxidation of isocitrate (Table 3*c*) and the activity of isocitrate lyase (Table 2*b*) were strongly reduced. Furthermore, the oxidation of isocitrate by purified mitochondria was markedly increased after the addition of purified glyoxysomes (Fig. 1).

DISCUSSION

The oxidation of citrate, *cis*-aconitate and isocitrate is ultimately achieved by the same enzyme, isocitrate dehydrogenase. The results presented in this paper show that in mitochondria isolated from *N. crassa* growing exponentially in a sucrose-containing medium, the rates of oxidation of citrate and *cis*-aconitate were rapid, whereas the rate of oxidation of

isocitrate was relatively slow. This is consistent with the rate of oxidation of citrate and *cis*-aconitate being directly limited by the activity of isocitrate dehydrogenase, while some other factor must restrict the rate of oxidation of isocitrate. Since both isocitrate dehydrogenases are located in the mitochondrial matrix, it is suggested that the slow rate of isocitrate oxidation could be due to a slow rate of translocation across the inner membrane. In rat liver mitochondria, the rate of oxidation of both *cis*-aconitate and isocitrate is limited by the rate of translocation through the inner membrane, while the rate of oxidation of citrate is limited by the activity of aconitate hydratase (Robinson & Chappell, 1970). The existence of transport systems for phosphate and dicarboxylate anions has been shown in mitochondria from *N. crassa* by Katkocin & Slayman (1976). However, there is no information available on the existence of transport mechanisms for tricarboxylate anions in these mitochondria. Further studies are therefore required to understand the operation and the specificity of tricarboxylate anion carrier(s) in *N. crassa* mitochondria. It could be useful to compare with the situation described for different species of *Enterobacteriaceae* (Imai, 1977, 1978). *Proteus mirabilis* and *Salmonella typhimurium* are able to oxidize external citrate, *cis*-aconitate and isocitrate, whereas *Proteus vulgaris* utilizes only citrate and *Escherichia coli* none of the tricarboxylates. Four different transport systems for the tricarboxylate anions have been described among these bacteria: the first system is specific for citrate and isocitrate, the second for *cis*-aconitate, the third for citrate, and the fourth for citrate and *cis*-aconitate. It is possible that *N. crassa* mitochondria also possess one or more tricarboxylate carriers with high affinities for citrate and *cis*-aconitate and a weak affinity for isocitrate.

The rates of oxidation of citrate and *cis*-aconitate decreased as the cultures aged. This may have been due to altered efficiency of the translocator of tricarboxylate anions. More probably, however, it may be related to alterations in the activity of the NADH dehydrogenase and in the efficiency of the first energy-coupling site, since the lowered rate of oxidation was accompanied by a lowered sensitivity to rotenone, a lowered ADP/O ratio and a loss of respiratory control.

The rate of oxidation of isocitrate increased as the cultures aged or when *N. crassa* was grown in an acetate-containing medium. The elevated rate of oxidation of isocitrate by washed mitochondrial pellets was resistant to inhibition by rotenone and sensitive to inhibition by malonate. Since the activity of isocitrate lyase was high in these preparations, it seems likely that the oxidation of isocitrate was not achieved via the mitochondrial isocitrate dehydrogenases, but occurred indirectly as the result of isocitrate being converted to succinate and glyoxylate in contaminating glyoxysomes.

The results presented here suggest a possible mechanism to control the equilibrium between the tricarboxylic acid cycle and the glyoxylate shunt by intracellular compartmentation. Since the citrate synthase was not found in glyoxysomes, these organelles would have to import a tricarboxylate substrate for isocitrate lyase. The specificity of the tricarboxylate anion carriers in the inner mitochondrial membrane could therefore be an important factor in regulating isocitrate breakdown.

This work was supported by the Swiss National Science Foundation (research grant 83.615.0.78 to J.P.S.). I.M.M. was the recipient of a NATO Science Fellowship and grants no. 511-15019 and 511-20033 from the Danish Natural Science Research Council.

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