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ISOCITRATE LYASE FROM *NEUROSPORA CRASSA*

I. PURIFICATION, KINETIC MECHANISM, AND INTERACTION WITH INHIBITORS

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SUMMARY

After establishing conditions which stabilize isocitrate lyase (EC 4.1.3.1) in cell-free preparations of *Neurospora crassa*, this enzyme was purified to homogeneity. The kinetic properties of the enzyme were studied in an effort to learn about the regulation of the enzyme in vivo, the mechanism of action and the properties of the active site. The enzyme was found to be inhibited by its products succinate and glyoxylate, and by phosphoenolpyruvate, fumarate, malate and fructose 1,6-bis-phosphate. The studies on the inhibition of the forward reaction by the enzyme products, glyoxylate and succinate, and studies of the kinetics of the back reaction showed that the enzyme functioned in a uni–bi reaction mechanism with succinate leaving before glyoxylate in the cleavage reaction. The inhibitions of the enzyme by maleate and the substrate analog homoisocitrate were examined, and the results shed light on the structure of the active site. In further studies, isocitrate lyase from both *N. crassa* and *Pseudomonas indigofera* was found to be competitively inhibited by the inorganic anions SO_4^{2-}, HPO_4^{2-}, Cl^- and NO_3^-. The results obtained suggest that the isocitrate lyases from *N. crassa* and *P. indigofera* may have a common evolutionary background.

INTRODUCTION

Isocitrate lyase (*threo*-D_s-isocitrate glyoxylate lyase, EC 4.1.3.1) is a key enzyme in an important, inducible anaplerotic pathway, the glyoxylate cycle. The substrate for the enzyme, isocitrate, is common to both the glyoxylate and Krebs cycles. The fate of isocitrate in metabolism determines whether carbon from acetyl-CoA is

Abbreviation: MOPS, morpholinopropane sulfonic acid.

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catabolized for the production of ATP and reducing potential by the Krebs cycle or whether carbon from acetyl-CoA is shunted into the anaplerotic [1] glyoxylate cycle for the replenishment of Krebs cycle intermediates. In this sense, the reaction catalyzed by isocitrate lyase is the first unique step in the glyoxylate cycle. As with other enzymes at the beginning of a metabolic pathway, it has been evident for some time that isocitrate lyase may be subject to feedback inhibition [2]. Investigations of isocitrate lyase from a number of different organisms have revealed inhibition by intermediates of both glycolysis and the Krebs cycle [2–9]. While results vary with the source of the enzyme, phosphoenolpyruvate [2–5], pyruvate [5, 6], fructose 1,6-bisphosphate [2], α-ketoglutarate [7, 8], malate [7, 8], and oxalacetate [5, 6, 8] have been shown to be inhibitors of isocitrate lyase. In addition, the reaction products of the enzyme, glyoxylate and succinate, have been shown to be significant inhibitors [2, 8, 9]. Thus, in general, the Krebs-cycle intermediates which are replenished by the anaplerotic glyoxylate cycle act as feedback inhibitors of isocitrate lyase as do some glycolytic intermediates.

In spite of its central importance in fatty acid metabolism, there is a paucity of information about isocitrate lyase from eukaryota. In the present study, an isocitrate lyase from a simple eukaryotic organism, *Neurospora crassa*, has been purified to homogeneity. The kinetics have been studied in some detail in order to determine how the enzyme may be regulated in the cell and to determine its mechanism of action. The results are described in this communication.

MATERIALS AND METHODS

Materials

The following unusual chemicals were obtained commercially: DL-isocitric acid·$1/2H_2O$ (*allo* free) A grade, ATP, ADP, AMP, rabbit muscle myokinase, morpholinopropane sulfonic acid (MOPS) [4], glutaric acid, and dithiothreitol from Calbiochem; fructose 1,6-bisphosphate, acetyl-CoA, dithiothreitol, nicotinamide adenine dinucleotide phosphate ($NADP^+$), succinic acid, sodium glyoxylate, and *threo*-D_S-isocitric acid from Sigma; phosphoenolpyruvate, L-malic acid, and DEAE-cellulose from Nutritional Biochemicals; L-aspartic acid and imidazole from Aldrich; L-glutamic acid, succinic acid, glycine, phenylhydrazine·HCl, and 2,6-dichlorophenol-indophenol from Eastman;fumaric acid from J. T. Baker; citric acid from Mallinckrodt; glyoxylic acid from Matheson, Coleman, and Bell; and Sephadex G-200, Sephadex G-50, and Blue Dextran from Pharmacia. Electrophoretically pure $NADP^+$-dependent isocitrate dehydrogenase from *Escherichia coli* was the kind gift of William F. Burke and Henry C. Reeves. All other organic and inorganic chemicals were of high purity.

Growth and isocitrate lyase induction

N. crassa, St. Lawrence strain 74A, was used and grown by a procedure based on one published by Flavell and Fincham [10]. The organism was first grown in 20 l of Vogel's minimal medium [11] supplemented with 1.5% sucrose. The medium was inoculated with conidia stocks that had been grown in two 250-ml Erlenmeyer flasks containing 30 ml Vogel's minimal medium, 1.5% sucrose, and 2% agar and was incubated at 31 °C with vigorous aeration. After about 22 h, at which time the growth

had reached late log phase, the mycelia were harvested by filtration with sterile cheesecloth, rinsed with Vogel's minimal medium containing 40 mM sodium acetate (pH 5.8), and then suspended in 20 l of the acetate medium. The mycelia were incubated in this medium at 31 °C with vigorous aeration for 6 h to induce the isocitrate lyase activity to high levels. The mycelia were then harvested, rinsed with distilled water, and squeezed to remove as much moisture as possible. The yield was 100–150 g.

Purification of isocitrate lyase

The column-elution buffers of the desired ionic strengths were mixed using appropriate amounts of the following stocks. Stock 1 contained 200 mM imidazole·HCl (pH 7.0) and 50.0 mM KCl. Stock 2 contained 10.0 mM EDTA (disodium salt), and 11.0 mM $MgSO_4 \cdot 7H_2O$; the pH of this stock was adjusted to 7.0 by the addition of NaOH. Dithiothreitol was added to the column elution buffer to a final concentration of 1.0 mM immediately before use.

Step I. Preparation of extract. The entire purification procedure was carried out at 3–5 °C. 100–150 g of *N. crassa* mycelia were ground with 1/4 mass of sand and 2.5 vol. of buffer in a mortar which had been chilled on ice. The buffer contained 0.4 M sucrose, 50 mM imidazole·HCl (pH 7.5), 10 mM KCl, 1.0 mM EDTA, 1.1 mM $MgSO_4$, and 1.0 mM dithiothreitol. After grinding, 2.5 vol. of additional buffer were added with stirring. The homogenate was filtered through cheesecloth and the filtrate centrifuged at 10 000 × *g* for 15 min.

Step II. $(NH_4)_2SO_4$ fractionation. The supernatant from the previous step was brought to 54% $(NH_4)_2SO_4$ saturation by the slow addition with continuous stirring of $(NH_4)_2SO_4$ solution saturated at 3 °C (pH approx. 7.0 with NH_4OH). The solution was then stirred continuously for at least 3 h at 5 °C. The precipitate was removed by centrifugation and discarded. The supernatant was then brought to 68% saturation by the addition of saturated $(NH_4)_2SO_4$, stirred as before, and the precipitate collected by centrifugation. The pellet was rinsed with about 30 ml of 68% saturated $(NH_4)_2SO_4$, and the precipitate collected by centrifugation.

Step III. Sephadex G-200. The pellet was taken up in about 10 ml of buffer (pH 7.0) containing 20 mM imidazole·HCl, 5.0 mM KCl, 1.1 mM $MgSO_4$, 1.0 mM EDTA, and 1.0 mM dithiothreitol. It was dialyzed against 10 vol. of the same buffer for 8–10 h to decrease the density sufficiently to prevent channeling on the Sephadex G-200 column. The dialyzed solution was loaded onto a 4.4 cm × 34 cm Sephadex G-200 column and the column was eluted with a 4-cm pressure head which gave a typical flow rate of 22 ml/h. Fractions of about 10 ml were collected and assayed for isocitrate lyase activity. All fractions with enzyme levels greater than 50% of the peak activity plus one additional fraction on each side of the peak were pooled.

Step IV. DEAE-cellulose column. The pooled fractions from the Sephadex G-200 column were placed on a 4.2 cm × 29 cm DEAE-cellulose column containing approx. 45 g of DEAE-cellulose. The column was eluted with about 600 ml of the same buffer as was used on the Sephadex G-200 column. The elution buffer was then changed to one (pH 7.0) with slightly higher ionic strength containing 28 mM imidazole·HCl, 7.0 mM KCl, 1.0 mM EDTA, 1.1 mM $MgSO_4$ and 1.0 mM dithiothreitol. The peak of isocitrate lyase activity was eluted with 600–800 ml of this higher ionic-strength buffer and the peak fractions were combined.

Step V. Concentration and storage. The combined fractions were concentrated

by pressure dialysis on a Diaflo ultrafilter to 5–8 ml and the concentrated enzyme was dialyzed against two changes of buffer (pH 6.8) containing 50 mM MOPS (sodium salt), 5.0 mM Mg^{2+}, 1.0 mM EDTA and 1.0 mM dithiothreitol. The dialyzed enzyme was then divided into small batches and stored in a frozen state at −80 °C.

Protein determination

Protein content was determined by the method of Lowry et al. [12]. The assay was standardized with fresh bovine serum albumin samples dissolved in the same buffer as the protein sample. In one case, amino acid analysis was used [13].

Electrophoresis

Analytical polyacrylamide gel electrophoresis was conducted by a slight modification of the procedure of Davis [14]. Gels were polymerized from both 7 and 4% acrylamide. The gels were prerun in gel buffer plus 2 mM mercaptoethanol as suggested by Mitchell [15]. The sample was placed directly on the running gel in 0.2 ml of buffer containing 5.98 g Tris, 48 ml of 1.0 M HCl, and 0.46 ml *N,N,N′,N′*-tetramethylenediamine per 800 ml. A drop of glycerol was added to each sample to aid in layering on the gel. The gels were stained with aniline black for 2 h and then destained by repeated changes of 7% acetic acid.

Sodium dodecylsulfate gel electrophoresis was carried out essentially as reported by Weber and Osborn [16].

Isocitrate lyase assays

The isocitrate lyase activity was assayed with five different procedures. In routine monitoring of enzyme activity, as during the isolation procedure and the molecular-weight determination, the activity was assayed by the method published by Kornberg [3] except that a final concentration of 1.25 mM dithiothreitol was used in place of glutathione, and the assay was run at 30.0 °C.

In the determination of the pH optimum, the isocitrate lyase activity was assayed at a single fixed concentration of 2 mM *threo*-D_s-isocitrate as described by Roche et al. [17] using some modifications by Ozaki and Shiio [8]. This concentration of isocitrate is known to be saturating at pH 6.8.

Throughout the kinetic studies, the following inorganic anion-free assay procedure was used. Stock buffer was made up by dissolving 100 mmoles MOPS and 2.0 mmoles EDTA in glass-distilled water. Then 10 mmoles Mg^{2+} were added as the $MgCO_3$ and the solution was stirred overnight using a magnetic stirrer to free it of CO_2. 16 mmoles Mg^{2+} were used in studies involving ATP, ADP, AMP and fructose 1,6-bisphosphate. The resulting solution was brought to pH 6.8 by the addition of NaOH, and then the volume was brought to 1.00 l.

Cl^--free phenylhydrazine was prepared by dissolving phenylhydrazine·HCl in water. The pH was adjusted to 8–10, the phenylhydrazine was extracted with diethyl ether, and the ether was evaporated.

A typical assay contained 0.625 ml stock buffer, 1.5 μmoles dithiothreitol, 4.4 μmoles phenylhydrazine, and substrate in a final volume of 1.25 ml. The assay was conducted at 30.0 °C. The substrate was always added last after temperature equilibrium had been established and a baseline had been run on the recorder. The change in the absorbance at 324 nm due to glyoxylate phenylhydrazone was followed

continuously on a Gilford 2400 recording spectrophotometer. The extinction coefficient of the glyoxylate phenylhydrazone is $1.68 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ [3]. One unit of enzyme is that amount which catalyzes the production of 1 μmole of glyoxylate per min under saturating conditions. Specific activity is: units/mg protein.

The condensation (reverse) reaction of isocitrate lyase was assayed using 0.375 ml stock buffer, 0.1 ml of the same buffer containing 0.26 units *E. coli* isocitrate dehydrogenase (K_m = 0.002 mM), 0.1 ml 5.9 mM $NADP^+$, and succinate, glyoxylate and water to give a volume of 0.95 ml. The reaction was started by the addition of 0.026 units of isocitrate lyase (as measured by the cleavage reaction) in 0.05 ml of 50 mM MOPS (sodium salt), 5.0 mM Mg^{2+}, 1.0 mM EDTA (pH 6.8) and 0.2 mM dithiothreitol. The assay was conducted at 30 °C. The change in the absorbance at 340 nm due to the formation of NADPH was followed continuously on a Cary 15 recording spectrophotometer.

When the inhibition of the enzyme by glyoxylate was being examined, the reaction rate was determined by measuring the succinate produced. The succinate was determined by a modification of the method of Rodgers [18, 19].

In the kinetic studies, all the metabolites and metabolite analogs that were tested were obtained either as the sodium salt or as the free acid. When they were obtained as the free acid, the pH was adjusted with NaOH.

The kinetic data were analyzed with the aid of a computer program, HYPER [20], or a similar program adapted for the Wang 700 (Number 1047A/GS2).

Energy charge

As reported by Klungsovr et al. [21] and Blair [22], an equilibrium mixture of ATP, ADP, and AMP was prepared using myokinase to catalyze the attainment of equilibrium.

RESULTS

Purification of the enzyme

In preliminary experiments, it was found that *N. crassa* isocitrate lyase in a crude 10 000 $\times$ *g* supernatant lost 30% of its activity in 2 days during storage at 4 °C, and lost all activity when frozen. Consequently, a large number of buffer systems were tested and two buffer systems were found in which purified enzyme lost less than half of its activity in 10 days at 4 °C [19]. The first buffer system contained 40 mM imidazole·HCl (pH 7.0), 10 mM KCl, 1.1 mM $MgSO_4$, 1.0 mM EDTA, and 1.0 mM dithiothreitol. The second buffer system contained 50 mM MOPS (sodium salt) (pH 6.8), 5.0 mM Mg^{2+}, 1.0 mM EDTA, and 1.0 mM dithiothreitol. In this second buffer system, the enzyme could be frozen at −80 °C for at least 6 months without measureable loss of activity or change in K_m. Utilizing these two buffer systems, a modified version of the isolation procedure published by Leckie and Fincham [23] was worked out as described in Materials and Methods.

Table I summarizes data obtained from a typical purification run. Overall, purified isocitrate lyase was obtained in a yield of 30% with a specific activity of 16. This is approximately a 1.5-fold better recovery than is obtained by Leckie and Fincham's procedure. In a later purification run in which the final protein concentration was determined by amino acid analysis, a specific activity of 30 was obtained.

TABLE I

SUMMARY OF A TYPICAL PURIFICATION RUN

Isolation Step	Total protein (mg)	Total units	Specific activity	Recovery (%)	Purification (-fold)
10 000 × *g* supernatant	3160	640	0.2	(100)	(1)
$(NH_4)_2SO_4$ fractionation	520	575	1.1	90	5.5
Sephadex G-200 column	90	340	3.8	53	19
DEAE-cellulose column	12.6	202	16.0	31	80

Isoenzyme purified

The isocitrate lyase that was purified was probably the isocitrate lyase-1 of Flavell and Woodward [24] for two reasons. It eluted off the DEAE-cellulose at about the same ionic strength as reported by Flavell and Woodward for isocitrate lyase-1 whereas isocitrate lyase-2 was reported to elute at about five times the ionic strength. Furthermore, a yield of 30% was obtained, while in Flavell and Woodward's work isocitrate lyase-2 accounted for no more than 20% of the total isocitrate lyase activity in the mycelium.

Criteria of purity

The final product was homogeneous by the criterion of disc gel electrophoresis (Fig. 1) in gels polymerized from both 7% and 4% acrylamide. Furthermore, no evidence of contamination was found on sodium dodecylsulfate–polyacrylamide gel electrophoresis.

Co-factor and buffer requirements

Isocitrate lyase from *N. crassa*, like all other isocitrate lyases that have been studied, required the presence of divalent metal ions such as Mg^{2+} for activity. It also required the presence of a thiol compound such as dithiothreitol in the assay buffer in order to show full activity. When the use of glass-distilled water and boiled glassware were combined with gel filtration to remove thiol, EDTA was partially effective in activating the enzyme.

pH optimum

The pH optimum of the *N. crassa* isocitrate lyase-1 was determined using the non-continuous assay. The optimum was found to be pH 6.8 with a sharp decrease in activity to pH 5.5. There was a 15% decrease in enzyme activity as the pH was increased to 7.7 and then a more rapid decrease in activity as the pH was increased to 9.5 [19].

Enzyme kinetic studies

Due to the great cost of the pure enzyme substrate, *threo*-D_S-isocitrate, it is common practice to use *threo*-DL-isocitrate in isocitrate lyase assays. The *threo*-L_S-

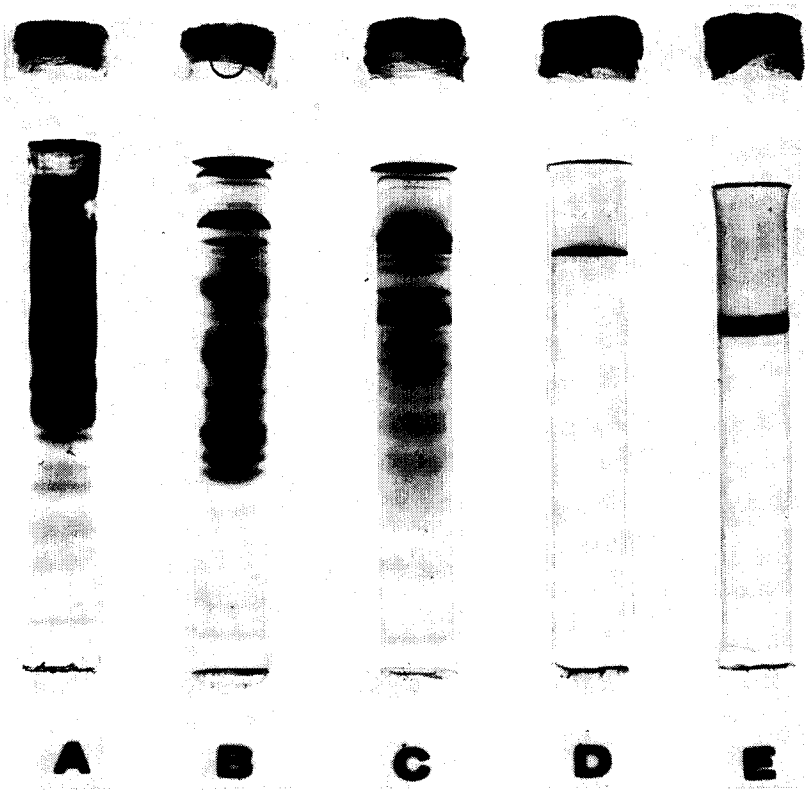


Fig. 1. Polyacrylamide gel electrophoresis showing the progress of the purification of isocitrate lyase. Gel A shows the 10 000 × *g* supernatant, B shows proteins in precipitate obtained with addition of $(NH_4)_2SO_4$ to 54–68% saturation, C shows the pooled fractions of the G-200 column, and D and E show the purified enzyme in gels polymerized from 7 and 4% acrylamide, respectively.

isocitrate has been shown to have no effect on the activities of the isocitrate lyases with which it has been tested [6]. In order to check the effect of *threo*-L_S-isocitrate on the kinetics of isocitrate lyase-1 from *N. crassa*, the K_m and V were determined for both *threo*-D_S-isocitrate and twice the concentration of *threo*-DL-isocitrate. Within experimental error, the presence of *threo*-L_S-isocitrate had no effect on the K_m and V. *threo*-DL-Isocitrate was used throughout the kinetic studies, and the concentration of the true substrate, *threo*-D_S-isocitrate, was used in all calculations and results.

A buffer system (pH 6.8) containing 50 mM MOPS (sodium salt), 5.0 mM Mg^{2+}, 1.0 mM EDTA, and 1.0 mM dithiothreitol was used throughout the kinetic studies. In this buffer system, a K_m of 0.05 mM was obtained for *threo*-D_S-isocitrate [19].

Inhibition by anions

Inorganic anions were found to competitively inhibit the purified isocitrate lyase from *N. crassa*. Detailed kinetic studies were conducted on the inhibition of the enzyme by the anions HPO_4^{2-}, SO_4^{2-}, Cl^-, NO_3^-, and acetate. As shown in the Lineweaver–Burk plot in Fig. 2 and the slope replot shown in the inset, the divalent

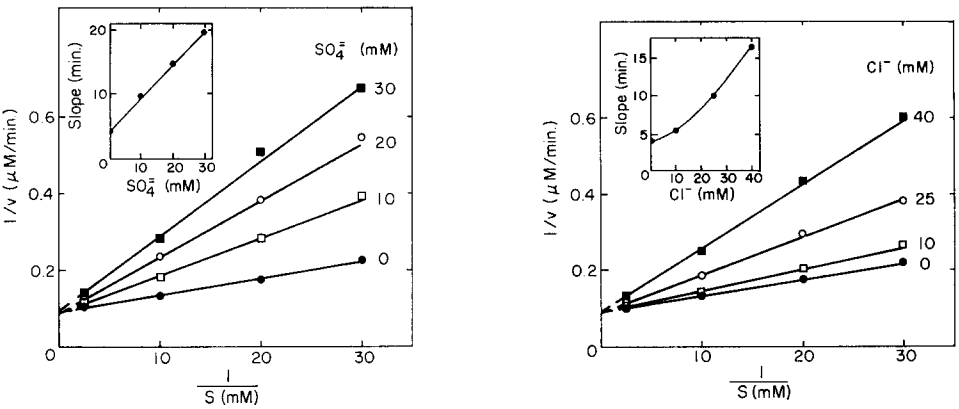


Fig. 2. Lineweaver–Burk plot showing the inhibition of *N. crassa* isocitrate lyase by varying levels of SO_4^{2-}. Each assay contained isocitrate, 62.5 μmoles MOPS, 6.25 μmoles Mg^{2+}, 1.25 μmoles EDTA, 1.5 μmoles dithiothreitol, and 4.4 μmoles phenylhydrazine in a final volume of 1.25 ml. See Materials and Methods for details. Also, each assay contained 0.011 unit/ml purified isocitrate lyase. A replot of the slopes vs the inhibitor concentrations is shown in the inset.

Fig. 3. Lineweaver–Burk plot showing the inhibition of isocitrate cleavage by varying Cl^-. Assay procedure was as in Fig. 2 with 0.011 unit/ml purified *N. crassa* isocitrate lyase. A replot of the slopes vs the inhibitor concentrations is shown in the inset.

anion, SO_4^{2-}, was a linear competitive inhibitor with respect to isocitrate. In contrast to this, the monovalent anions, Cl^-, NO_3^-, and acetate are non-linear competitive inhibitors with the shape of the slope replots being concave upwards. The results obtained for the Cl^- inhibition, as shown in the Lineweaver–Burk plot in Fig. 3 and the slope replot in the inset, are typical of the results obtained with monovalent anions. When the slopes for the monovalent anions are replotted against the squares of the substrate concentrations, a straight line is obtained for the NO_3^- inhibition whereas the lines obtained for the Cl^- and acetate inhibitions are concave downwards.

Under the assay conditions at pH 6.8, phosphate is present as two different major species, the monovalent anion, $H_2PO_4^-$, and the divalent anion, HPO_4^{2-}. The inhibition by the phosphate was linearly competitive, which indicates by analogy to the effects of SO_4^{2-} that it is most likely the divalent anion species, HPO_4^{2-}, that is the predominant inhibitor of the enzyme.

The inhibitory effects of HPO_4^{2-}, SO_4^{2-}, Cl^-, and NO_3^- on the *Pseudomonas indigofera* isocitrate lyase were also examined. As with the *N. crassa* isocitrate lyase, the divalent anions, HPO_4^{2-} and SO_4^{2-}, were linear competitive inhibitors of the *P. indigofera* isocitrate lyase, and the monovalent anions, Cl^- and NO_3^-, were non-linear competitive inhibitors with the shapes of the slope replots being concave upwards. The most notable difference was the fact that the slope replots for the monovalent anions were not as sharply concave upwards for the *P. indigofera* enzyme.

A summary of the data on the anion inhibitors of both isocitrate lyases is shown in Table II. The percentage inhibition by 20 mM of each anion at a concentration of *threo*-D_s-isocitrate which is two times the K_m value is shown. The K_i values for HPO_4^{2-} and SO_4^{2-} were determined from the slope replots and found to be 1.5 and 9.0 mM, respectively, for the *N. crassa* enzyme and 1.8 and 13 mM, respectively, for the *P. indigofera* enzyme. The K_i values for the inhibitions by Cl^-, NO_3^- and

TABLE II

ANION INHIBITION OF THE ISOCITRATE LYASES FROM *N. CRASSA* AND *P. INDIGOFERA*

Assay conditions were as described in Fig. 2. The respective K_m values for *threo*-D_S-isocitrate under the assay conditions for the *N. crassa* and *P. indigofera* isocitrate lyases were 0.05 and 0.013 mM; two times half-saturating substrate concentrations of 0.1 mM and 0.025 mM, respectively, were used with the two enzymes. The inhibition by 20 mM of each anion inhibitor (except 9 mM HPO_4^{2-}) is shown for each enzyme.

	HPO_4^{2-}	SO_4^{2-}	Cl^-	NO_3^-	Acetate
Percentage inhibition *N. crassa*	65	43	20	22	10
Percentage inhibition *P. indigofera*	64	29	9	16	–
K_i (mM) *N. crassa*	1.5	9.0	—	—	—
K_i (mM) *P. indigofera*	1.8	13	—	—	—

acetate were not calculated since the data obtained were insufficient to establish a clear mechanism of inhibition. As can be seen in the table, the divalent anions inhibit the two enzymes nearly equally whereas the monovalent anions inhibit the *N. crassa* isocitrate lyase somewhat more strongly.

Inhibition by metabolites and metabolite analogs

The effects of a number of metabolites and metabolite analogs on the cleavage reaction of isocitrate lyase-1 were investigated. In the presence of 0.05 mM isocitrate, the inhibitions by 5.0 mM glycine, aspartate, or glutamate, or 0.1 mM acetyl-CoA were 12, 2, 1 and 15%, respectively. These inhibitions were considered to be insignificant and were not explored further. In additional experiments, the energy charge

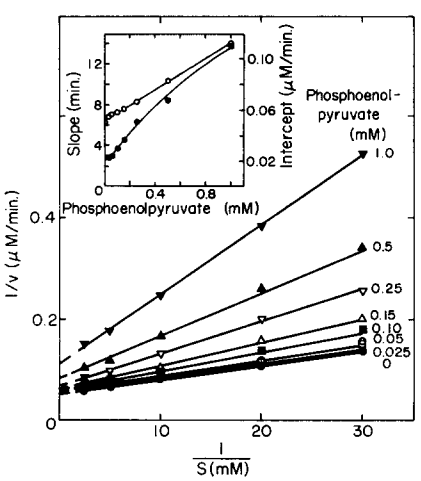


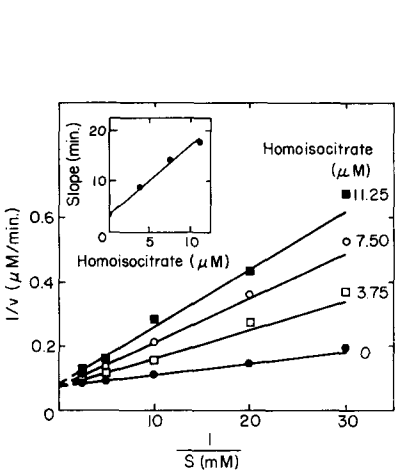
Fig. 4. Lineweaver–Burk plot showing the inhibition of isocitrate cleavage (0.019 unit/ml purified isocitrate lyase) by phosphoenolpyruvate. Assay procedure was as described in Fig. 2. The inset shows replots of the slopes (●) and intercepts (○).

resulting from varying ratios of ATP, ADP and AMP was not found to have a significant effect on the activity of the enzyme [19].

Outside of the enzyme products, succinate and glyoxylate, phosphoenolpyruvate was the strongest inhibitor of the metabolites tested. The Lineweaver–Burk plot for the phosphoenolpyruvate inhibition is shown in Fig. 4. As can be seen in the plot, the inhibition at very low phosphoenolpyruvate concentrations appears uncompetitive. At higher phosphoenolpyruvate concentrations, the inhibition is clearly noncompetitive [25]. The slope and intercept replots, as shown in the inset, are, respectively, slightly concave downwards and almost linear. From the slope replot, a K_i of 0.2 is obtained.

Fumarate was found to be a linear competitive inhibitor of the enzyme (not shown). Extrapolation of the slope replot gave a K_i of 1.0 mM. Malate and fructose 1,6-bisphosphate were both linear noncompetitive inhibitors of the enzyme (also not shown) with K_i values of 1.3 and 1.5 mM, respectively.

The substrate analog, *threo*-DL-homoisocitrate, was found to be an exceedingly strong linear competitive inhibitor of the enzyme, as shown in Fig. 5. Extrapolation of the slope replot gives a K_i of 0.003 mM. There was no detectable cleavage of *threo*-DL-homoisocitrate to glyoxylate under conditions which would have measured



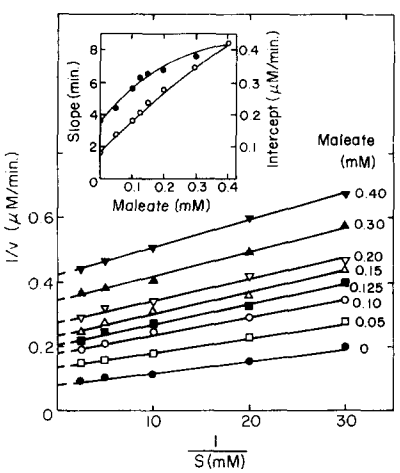
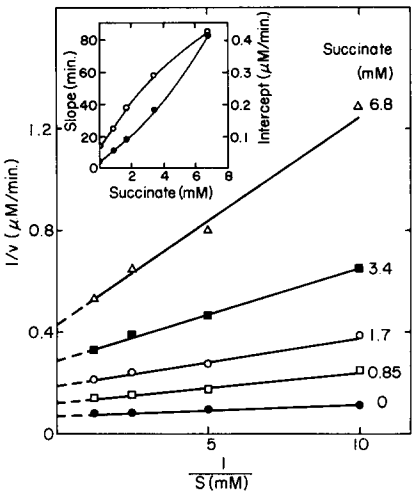


Fig. 5. Lineweaver–Burk plot showing the inhibition of cleavage (0.013 unit/ml purified isocitrate) by the substrate analog *threo*-DL-homoisocitrate. Assay procedure was as specified in Fig. 2. The inset shows a replot of the slopes.

Fig. 6. Lineweaver–Burk plot showing the inhibition of isocitrate cleavage (0.012 unit/ml purified isocitrate lyase) by maleate. Assay procedure was as described in Fig. 2. The inset shows replots of the slopes (●) and intercepts (○).

0.1% of the rate for *threo*-D_s-isocitrate. Cleavage could only be detected at very high enzyme concentrations and the maximal velocity was 1/1600 of that for isocitrate.

The inhibition of the enzyme by maleate, as shown in Fig. 6, appears to be S-2/1 I-hyperbolic in the terminology of Cleland [25] suggesting that maleate can be bound either by free enzyme or the enzyme–glyoxylate complex.



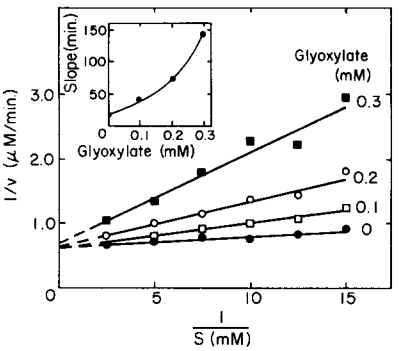


Fig. 7. Lineweaver–Burk plot showing the inhibition of isocitrate cleavage (0.014 unit/ml purified isocitrate lyase) by succinate. Assay procedure was as specified in Fig. 2. The inset shows replots of the slopes (●) and intercepts (○).

Fig. 8. Lineweaver–Burk plot of the inhibition of isocitrate cleavage (0.0016 unit/ml purified isocitrate lyase) by glyoxylate. The reaction velocities were determined by measurement of the succinate produced. The inset shows a replot of the slopes.

Product inhibition of the enzyme

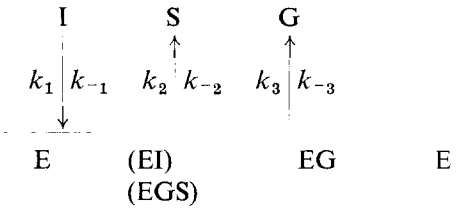
Both of the enzyme products, succinate and glyoxylate, were strong inhibitors. Succinate at lower concentrations (Fig. 7) was a linear noncompetitive inhibitor. The slope and intercept replots are linear for succinate concentrations up to 1.7 mM. At concentrations of succinate greater than 1.7 mM the replots become non-linear. Extrapolation of the linear portion of the slope replot to zero slope gives a K_i of 0.50 mM. Consequently, the slope and intercept replots are linear for concentrations of succinate up to more than three times the binding constant (K_i) for succinate. As shown in Fig. 8, glyoxylate was a competitive inhibitor of the enzyme. The replot of the slopes is concave upwards, but it is nearly linear at low glyoxylate concentrations. Extrapolation of the slope replot between 0.0 and 0.1 mM glyoxylate gives a K_i of 0.07 mM.

Condensation (reverse) reaction of the enzyme

The condensation reaction of the enzyme was examined at varying concentrations of both glyoxylate and succinate. Substrate inhibition was observed for glyoxylate concentrations above 0.2 mM and succinate concentrations above 1.0 mM. The intercept replots were linear with the reciprocal of concentrations (not shown) in concentration ranges of 0.067–0.2 mM glyoxylate and 0.25–1.0 mM succinate. The K_m values for glyoxylate and succinate derived from the intercept replots were 0.14 and 0.44 mM, respectively.

Postulated mechanism

Based on the data for the cleavage and condensation reactions of the enzyme, we propose the following mechanism in which product release is ordered:



threo-D_S-Isocitrate, succinate, and glyoxylate are represented by I, S, and G, respectively.

DISCUSSION

In the present study, the addition of a thiol-protective reagent to the assay buffer was required for the enzyme to show full activity. When heavy-metal ion contamination of reagents had been reduced, EDTA gave partial activation of *N. crassa* isocitrate lyase. In earlier experiments fresh enzyme from *P. indigofera* was found to be fully activated by EDTA. It was suggested that thiol reagents activate isocitrate lyase by reducing the enzyme to a catalytically active species [26, 27] and removing inhibitory heavy metal ions [26–29]. Only in the latter role can a thiol be replaced by EDTA. The presently reported partial activation by EDTA suggests that the thiol reagent may be serving in part to remove heavy metal ions which inhibit the enzyme.

The present kinetic studies of isocitrate lyase from *N. crassa* provide information about the possible mode of regulation of the enzyme in the cell, about the mode of catalysis by the enzyme, and about the structure of the active site of the enzyme.

N. crassa isocitrate lyase was found to be inhibited by succinate, L-malate, fumarate, phosphoenolpyruvate, and fructose 1,6-bisdiphosphate. These results indicate that the activity of the isocitrate lyase from *N. crassa* is inhibited in vivo when the concentrations of certain Kreb cycle and/or glycolytic intermediates increase. In this way, *N. crassa* would utilize the anaplerotic glyoxylate cycle only when these intermediates were present at lower levels.

Linear double-reciprocal plots were obtained for the condensation reaction when both glyoxylate and succinate were varied up to about twice their respective K_m values. This indicates that the condensation reaction occurs either by an ordered binding or random binding of substrates in rapid equilibrium with the enzyme. In studies of the cleavage reaction, the competitive inhibition by glyoxylate and the linear noncompetitive inhibition by lower concentrations of succinate support an ordered binding mechanism for condensation in which glyoxylate is bound first with subsequent binding of succinate [25]. Thus the kinetic mechanism is the same as that for the enzyme from *P. indigofera* [9]. In the present work, the upward concavity of slope replots for inhibition of the cleavage reaction at higher concentrations of glyoxylate and succinate and the downward concavity of intercept replots for succinate suggest that the condensation reaction becomes more random in these concentration ranges [25]. In contrast, the strictly linear competitive inhibition of isocitrate cleavage catalyzed by the enzyme from *P. indigofera* with concentrations of glyoxylate up to 0.6 mM suggests that the reaction catalyzed by the bacterial enzyme is less random at higher concentrations of glyoxylate and succinate [9].

The present results with inhibitors of the *N. crassa* isocitrate lyase are similar to those obtained by McFadden and co-workers in studies of the enzyme from *P. indigofera* [2, 7, 9]. With the enzyme from *P. indigofera*, phosphoenolpyruvate was a strong inhibitor which showed uncompetitive inhibition at low concentrations and noncompetitive inhibition at higher concentrations. Maleate was also a strong inhibitor. In less detailed studies, malate, fumarate, and fructose 1,6-bisphosphate were found to be weak inhibitors; and glycine, aspartate, glutarate, and acetate did not inhibit the enzyme.

A number of much less detailed studies have been conducted on other isocitrate lyases. The results of kinetic studies on the isocitrate lyase from *Pseudomonas aeruginosa* [30] are consistent with catalysis by rapid-equilibrium random or ordered reaction mechanisms like that by the *P. indigofera* enzyme. The isocitrate lyases from *Bacillus flavum* [8], *Clostridium pyrenoidosa* [5] and *Pinus pinea* [4] are inhibited by several of the same metabolites as the *N. crassa* and the *P. indigofera* enzymes. The enzyme from *E. coli* is inhibited by phosphoenolpyruvate [3]. From the available data, these isocitrate lyases may be similar to the *N. crassa* and *P. indigofera* isocitrate lyases.

Small anions were found to inhibit both the *P. indigofera* and the *N. crassa* isocitrate lyases in the same manner. The anion-inhibition data that were obtained with SO_4^{2-}, HPO_4^{2-}, Cl^-, NO_3^-, and acetate are compatible with the masking of two adjacent sites by the divalent anions and the multiple binding of two monovalent anions to the adjacent sites. The adjacent sites may be two ligand-binding sites for Mg^{2+} which is thought to interact with the glyoxylate at the active site [29]. The effect of this anion inhibition in vivo might be to decrease the binding by the enzyme of the substrate, *threo*-D_s-isocitrate. Thus, the effective in vivo binding constant for the substrate may not be as low as has been determined from in vitro studies.

The inhibitions of the isocitrate lyase from *N. crassa* by maleate and fumarate have intriguing implications with regard to the structure of the active site. The strong inhibition by maleate in comparison to the weaker inhibition by fumarate suggests that succinate indeed binds to the enzyme in a *cis*-configuration [30]. The linear competitive inhibition by fumarate perhaps suggests that it binds to the glyoxylate-binding portion of the active site.

The exceedingly strong competitive inhibition of the enzyme by homoisocitrate indicates that it binds to the active site much more tightly than the substrate, isocitrate. With its extra length between the β- and terminal carboxyl groups, the homoisocitrate appears to fit into the active site better than the substrate. This suggests that along the reaction path, the analogous carboxylates of isocitrate assume a strained *cis*-configuration coincident, perhaps, with carbon–carbon bond weakening.

The kinetic evidence presented here suggests that the isocitrate lyases from *N. crassa* and *P. indigofera* may have a common evolutionary background. The structural studies presented in the companion paper [31] lend further support to the conclusion that the two isocitrate lyases have evolved from a common origin.

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