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ISOCITRATE LYASE FROM *NEUROSPORA CRASSA*

II. COMPOSITION, QUATERNARY STRUCTURE, C-TERMINUS, AND ACTIVE-SITE MODIFICATION

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SUMMARY

Several features of the molecular structure of *Neurospora crassa* isocitrate lyase (*threo*-D_s-isocitrate glyoxylate lyase, EC 4.1.3.1) were studied. The subunit molecular weight was found to be 67 000 by the procedure of Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412. The molecular weight of the native enzyme was indicated to be 270 000 by gel filtration. These values suggest that the protein is tetrameric. The enzyme was found to have a histidine in the C-terminal position and a phenylalanine in the penultimate position. The C-terminal amino acids were very important for enzyme activity. 3-Bromopyruvate was found to irreversibly inactivate the enzyme with the inactivation showing saturation kinetics. Substrate or products protected against the inactivation. The amino acid content was also determined. The *N. crassa* isocitrate lyase was, in most characteristics, similar to the *Pseudomonas indigofera* isocitrate lyase. The overall similarities provide a basis for suggesting that the prokaryotic *P. indigofera* isocitrate lyase is homologous to the eukaryotic *N. crassa* isocitrate lyase.

INTRODUCTION

Isocitrate lyase has been shown to be present in a wide range of organisms including bacteria, green algae, protozoa, fungi, plants and some animals (for reviews see refs 1–4). In most cases, isocitrate lyase functions as part of the anaplerotic glyoxylate cycle which these organisms utilize to replenish Krebs-cycle intermediates when growing or differentiating on acetate or substrates which are catabolized principally to acetyl-CoA [1].

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Abbreviation: MOPS, morpholinopropane sulfonic acid.

All of the isocitrate lyases that have been studied have an absolute requirement for a divalent metal ion for activity. Optimal activities are obtained with Mg^{2+}. Isocitrate lyases have a high specificity for *threo*-D$_s$-isocitrate. No cleavage has been detected with citrate, *cis*-aconitate, *threo*-L$_s$-isocitrate, or *threo*-L$_s$-isocitrate lactone [5, 6–8] nor has evidence been found for condensation of compounds other than glyoxylate and succinate in detailed studies of the back reaction [9]. However, the substrate analog α-methylisocitrate is cleaved slowly by isocitrate lyase from *Pseudomonas indigofera*, *Neurospora crassa*, *Saccharomyces cerevisiae* and *Chlorella vulgaris* [10].

A detailed study of the kinetics of the *Neurospora crassa* isocitrate lyase was presented in the companion paper to this one [11]. In that study, the *N. crassa* isocitrate lyase was found to function by the same kinetic mechanism of action, and to be regulated by the same metabolites as have been reported for *P. indigofera* isocitrate lyase. To the extent that other isocitrate lyases have been studied [12–15], their regulation is similar to the *P. indigofera* and *N. crassa* isocitrate lyase.

Several characteristics of the molecular structure of *P. indigofera* isocitrate lyase have been studied by McFadden and coworkers [3, 16–18] and there is some limited information available on the molecular weight of several isocitrate lyases from other sources [1, 19, 20]. Beyond these descriptions there is a paucity of information on the structure of different isocitrate lyases. We now report some of the structural characteristics of an isocitrate lyase from a simple eukaryote, *N. crassa*.

MATERIALS AND METHODS

Materials

In addition to the unusual chemicals mentioned in the companion paper, the following were obtained commercially: 3-bromopyruvate [4] from Calbiochem; egg white lysozyme, rabbit muscle aldolase, rabbit muscle phosphorylase *a*, and jack bean urease (55 000 units/g) from Sigma; bovine γ-globulin, and bovine serum albumin from Pentex; beef liver catalase from Mann; pig heart muscle fumarase from Nutritional Biochemicals; and dinitrofluorophosphate-treated carboxypeptidase A from Worthington. All other organic and inorganic chemicals were of high purity. *N. crassa* isocitrate lyase was prepared as described in the companion paper [11].

Molecular weight by gel filtration

Molecular weights were estimated by gel filtration on a Sephadex G-200 column by a method based on the work of Andrews [21]. A 1.0 cm $\times$ 54 cm Sephadex G-200 column was packed, equilibrated, and eluted with buffer (pH 7.0) containing 2 mM imidazole·HCl, 150 mM KCl, 1.0 mM EDTA, 1.1 mM $MgSO_4$ and 0.5 mM dithiothreitol. Fractionation was calibrated with Blue Dextran, jack bean urease, fumarase, and bovine serum albumin because these standards gave a good fit on the curve in plots of V_e/V_o vs log molecular weight in Andrews' work [21].

The fumarase activity was measured by a modification of the method of Kanarek and Hill [22]. The final assay mixture contained 20 mM imidazole·HCl, 5.0 mM KCl, 50 mM L-malate (pH 7.0) and enzyme. The assay was run at 30 °C and the initial increase in the extinction at 230 nm caused by the α,β-unsaturated acid,

fumarate, was measured on a Gilford 2400 recording spectrophotometer. The extinction coefficient of fumarate at 230 nm was $4.64 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$.

The assay for urease was similar to the one used by Andrews [21]. As published, the assay would not work in our hands. However, when 1.0 mM dithiothreitol was substituted for 1.25 mM EDTA, the assay worked well. Using this change, urease was assayed on a Sargent pH stat in a solution containing 50 mM KCl, 50 mM urea, and 1.0 mM dithiothreitol. The net base formed by the hydrolysis of the urea at a pH of 7.0 was titrated with 10 mM HCl. The Blue Dextran and bovine serum albumin peaks were determined by measuring their absorbances at 625 nm and 280 nm, respectively.

Sodium dodecylsulfate gel electrophoresis

The molecular weight of the isocitrate lyase subunits was determined by sodium dodecylsulfate gel electrophoresis essentially as reported by Weber and Osborn [23]. The protein samples were pretreated by incubating at 37 °C for 2 h in 10 mM sodium phosphate buffer (pH 7.0), containing 0.1% sodium dodecylsulfate and 0.1% mercaptoethanol. The gels were standardized with phosphorylase *a*, bovine serum albumin, catalase, fumarase, isocitrate lyase from *P. indigofera* (kindly supplied by J. O. Williams) and aldolase.

Amino acid analysis

Samples of approx. 0.5 mg of isocitrate lyase were placed in vials with 1.0 ml of constant-boiling (5.7 M) HCl. The vials were immediately flushed with nitrogen, evacuated, and sealed. The samples were hydrolyzed at 110 °C for 12, 24 and 48 h. The amino acid analysis with norleucine as an internal standard was carried out on a Beckman Spinco 120C Automatic amino acid analyzer by the method of Moore and Stein [24]. Half-cystine was determined as cysteic acid after HCO_3H oxidation at 50 °C by the method of Hirs [25]. The tryptophan content was estimated by the method of Goodwin and Morton [26] from the ultraviolet absorptions at 294.4 and 280 nm of the enzyme dissolved in 0.1 M NaOH. The serine and threonine contents were estimated by extrapolation back to zero time of hydrolysis. The results obtained after 48 h hydrolysis were used as the values for valine, isoleucine, and leucine. The results for all the other amino acids were averaged.

Carboxypeptidase A determination of C-terminal amino acids

Isocitrate lyase was treated with carboxypeptidase A by a procedure based on the methods described by Ambler [27]. 1 mg of carboxypeptidase A crystals were rinsed in 5 ml of buffer (pH 8.0) containing 50 mM morpholinopropane sulfonic acid (MOPS) (sodium salt), 5.0 mM Mg^{2+}, and 1.0 mM EDTA and centrifuged down. The rinsed crystals were taken up in 4–10 ml of the same buffer containing 10% NaCl. Undissolved residue was centrifuged down. Isocitrate lyase stocks of 0.7–1.4 mg/ml were prepared in 3.25 ml of buffer (pH 8.0) containing 50 mM MOPS (sodium salt), 5.0 mM Mg^{2+}, 1.0 mM EDTA and 1.0 mM dithiothreitol. In order to determine protein concentration, a hydrolysis and an amino acid analysis [24] with norleucine as an internal standard were performed on a 0.25-ml sample of the stock. Norleucine was added to the remaining isocitrate lyase stock as an internal standard.

For a zero time sample, 0.5 ml of isocitrate lyase stock and 0.15 ml of the

carboxypeptidase stock were each added directly to 0.65 ml of 10% trichloroacetic acid. For the remaining studies 0.75 ml of the carboxypeptidase stock was added to the remaining isocitrate lyase stock and the mixture was incubated at 30 °C. Samples were withdrawn at intervals and added to an equal volume of 10% trichloroacetic acid. Simultaneously samples were withdrawn, and immediately diluted and assayed for enzyme activity at a final dilution of 1200 to 1. The trichloroacetic acid-treated samples were centrifuged to remove the precipitated protein, the pellets rinsed with 5% trichloroacetic acid, and the supernatants combined for each sample. After further acidification with 6 M HCl, the supernatants were extracted twice with diethyl ether. The extracted supernatants were dryed in vacuo over NaOH and taken up in citrate buffer. The amino acid contents of the samples were determined on a Beckman Spinco 120 C Automatic amino acid analyzer equipped with the range expander card and long path cuvettes.

Hydrazinolysis

Hydrazine was prepared and hydrazinolysis was carried out on 1 mg of isocitrate lyase by the methods of Schroeder [28]. The techniques of Korenman et al. [29] were used for separating the amino acids from the hydrazides. The amino acids were determined by amino acid analysis as described above [24].

Inactivation by 3-bromopyruvate

In order to remove the dithiothreitol which was present in the enzyme storage buffer, the enzyme was gel filtered on a Sephadex G-50 column equilibrated in 50 mM MOPS (sodium salt), 5.0 mM Mg^{2+}, and 1.0 mM EDTA (pH 6.8). The inactivation study was carried out in the same buffer. Appropriate amounts of 3-bromopyruvate and other compounds were added to give the desired concentration. The inactivation reaction at 30 °C was stopped after the desired time interval by the addition of sufficient dithiothreitol to give a concentration of 5.0 mM. After 10 min, isocitrate was added to a concentration of 2.0 mM and the assay for isocitrate lyase activity was continued as described by Roche et al. [30] using some modifications by Ozaki and Shiio [13]. In those studies in which protection of the enzyme by substrate and products was examined, the test compounds were removed by gel filtration on a Sephadex G-50 column prior to assaying for enzyme activity.

Isocitrate lyase assays

All isocitrate lyase assays used were as described in the companion paper [11].

RESULTS

Molecular weight

The molecular weight of the *N. crassa* isocitrate lyase in a crude 10 000 × *g* supernatant was determined as described in Materials and Methods. Immediately after the column had been calibrated, 0.6 ml of crude supernatant containing 0.5 units of isocitrate lyase and Blue Dextran were run through the column. A plot of the K_{av} values $\left(K_{av} = \frac{V_e - V_o}{V_t - V_o}\right)$ vs the molecular weights is shown in Fig. 1. A molecular

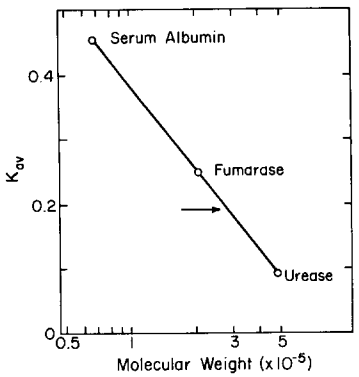

Fig. 1. Plot of the K_{av} values of the standards on a Sephadex G-200 column vs their molecular weights (on a log scale). Urease has a molecular weight of 490 000, fumarase a molecular weight of 205 000, and bovine serum albumin a molecular weight of 68 000. The K_{av} of the *N. crassa* isocitrate lyase, 0.192, is indicated by the arrow, and corresponds to a molecular weight of 270 000.

weight of 270 000 was obtained for the isocitrate lyase making the usual assumption that the test protein and proteins used as standards have similar shapes and degrees of hydration. In this connection, proteins with frictional ratios (f/f_o) from 0.75 to 1.34 [31] have been shown to give a good fit to a line when their elution volumes are plotted against the log of the molecular weights [21]. Isocitrate lyases from *C. pyrenoidosa* and *P. indigofera* have frictional ratios of 1.25 [19] and 1.38 [17], respectively. It seems reasonable to assume that a good estimate of the molecular weight of the *N. crassa* isocitrate lyase has been obtained in the present work with the standards employed [21].

Subunit molecular weight

The molecular weight of the isocitrate lyase subunits was determined by sodium dodecylsulfate gel electrophoresis [23]. The mobilities of a number of standards were determined and graphed against the subunit molecular weights (see Fig. 2).

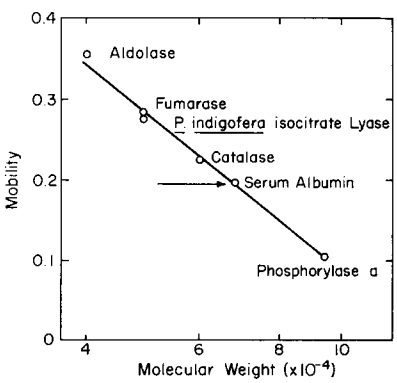

Fig. 2. Plot of the mobilities of the standards after sodium dodecylsulfate treatment and gel electrophoresis vs their subunit molecular weights. The arrow indicates the mobility of the *N. crassa* isocitrate lyase subunits, 0.200. This corresponds to a subunit molecular weight of 67 000.

It is significant that the subunit molecular weight of one of these standards, isocitrate lyase from *P. indigofera*, was 48 000 as measured in previous work by sedimentation equilibrium centrifugation in the presence of guanidinium chloride or urea [16], and that the mobility observed in Fig. 2 was in excellent agreement with that anticipated. The mobility of the *N. crassa* isocitrate lyase subunits, as indicated by the arrow, was essentially the same as the mobility of bovine serum albumin. From these results, a subunit molecular weight of 67 000 was obtained for the *N. crassa* isocitrate lyase. This subunit molecular weight of 67 000 is very close to one-fourth of the molecular weight of 270 000 that was indicated for the whole enzyme suggesting that in one stable form the enzyme is tetrameric.

Amino acid composition

The amino acid composition of *N. crassa* isocitrate lyase was determined and the results are shown in Table I. The recovery of the protein mass on the basis of the mass of the amino acid residues recovered was 85%. As can be seen in the table, the amino acid mole percentages in the *N. crassa* and the *P. indigofera* isocitrate lyases are strikingly similar. The mole percentages of aspartic acid, glutamic acid, proline and arginine in the two enzymes differ by less than 5%, and the mole percentages of the other amino acids are generally close.

Marchalonis and Weltman [32] have compared the amino acid contents of different proteins by using the function $S/1Q = S(X_{i,j} - X_{k,j})^2$. X_j is the content of a

TABLE I

AMINO ACID COMPOSITION OF *N. CRASSA* ISOCITRATE LYASE

Amino acid	Mole percentage in *N. crassa* isocitrate lyase	Residue per molecule of *N. crassa* isocitrate lyase*	Mole percentage in *P. indigofera* isocitrate lyase
Aspartic acid	10.09	246	9.66
Threonine	5.25	128	5.86
Serine	4.94	120	4.39
Glutamic acid	12.30	300	11.77
Proline	3.83	93	3.93
Glycine	6.45	157	8.61
Alanine	12.13	296	13.02
Half-cystine	1.36	33	1.03
Valine	5.33	130	7.63
Methionine	2.36	58	2.09**
Isoleucine	5.15	126	4.07
Leucine	8.07	197	6.85
Tyrosine	3.33	81	4.10
Phenylalanine	2.86	70	3.46
Lysine	7.91	193	5.59
Histidine	1.82	44	2.28
Arginine	4.65	113	4.61
Tryptophane	2.16	53	1.05

* This assumes a molecular weight of 270 000.

** Methionine was determined as the methionine sulfone.

given amino acid of type j expressed as residues per 100 residues or mole percentage. The subscripts i and k identify the particular proteins which are being compared. Half-cystine and tryptophan were not included by these authors in the computation of the $S\Delta Q$ values. Values in the region between 0 and 100 were obtained for pairs of homologous proteins and values in the region between 100 and 2000 were obtained for pairs of proteins which were believed to be unrelated. When the amino acid compositions of the isocitrate lyases from *N. crassa* and *P. indigofera* were compared, $S\Delta Q = 22$. This is well within the range of values expected for related or homologous proteins.

C-Terminal amino acids

When the enzyme was digested by carboxypeptidase A, histidine and phenylalanine were released first in about equal amounts (see Fig. 3). After hydrazinolysis, histidine was recovered, but no phenylalanine was recovered. Since carboxypeptidase

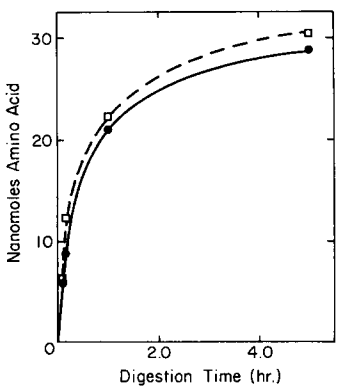

Fig. 3. Amino acid release from 14 nmoles *N. crassa* isocitrate lyase upon treatment with carboxypeptidase A showing histidine release (●—●) and phenylalanine release (□---□). Procedure was as in Materials and Methods.

A-catalyzed release of phenylalanine is known to be considerably more rapid than that of histidine, the results are consistent with histidine being the C-terminal residue (as established by hydrazinolysis), and with phenylalanine being the penultimate residue. In the experiments with a high ratio of carboxypeptidase A to isocitrate lyase, maximal yields of histidine and phenylalanine ranged from 82 to 100% of theoretical for a tetrameric structure. Zero time controls in which isocitrate lyase and carboxypeptidase A were added directly to trichloroacetic acid showed that the two protein preparations were free from amino acids. Controls in which carboxypeptidase A blanks were incubated during the C-terminal analysis of isocitrate lyase showed that there was no measurable autodigestion of the carboxypeptidase A during the experiment.

As shown in Fig. 4, enzyme activity was lost as the C-terminal histidine was cleaved. Activity loss was in close accord with the histidine removal as indicated by the concordance between the theoretical decay and the experimental data. This indicates that the C-terminal residues are in some way essential for enzyme activity.

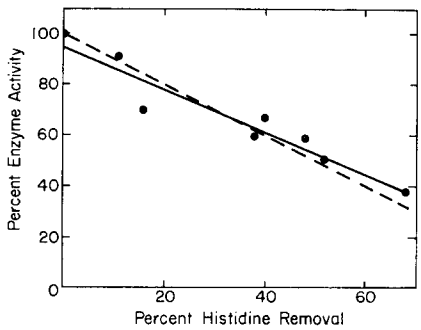

Fig. 4. Loss of activity with carboxypeptidase A catalyzed removal of histidine. Results shown are a composite of two different experiments. Dashed line (– – –) shows theoretical values.

Inactivation by 3-bromopyruvate

3-Bromopyruvate has been shown to irreversibly inactivate *P. indigofera* isocitrate lyase by alkylating a cysteine residue at the active site [18]. The effect of 3-bromopyruvate on *N. crassa* isocitrate lyase was examined. The inactivation of the enzyme by varying concentrations of 3-bromopyruvate in the presence of 1.0 mM EDTA is shown in Fig. 4. In the absence of EDTA, it was necessary to use 3-bromopyruvate concentrations of at least 2 mM in order to obtain detectable inactivation of the enzyme.

The data in Fig. 5 can be analyzed in the following manner [18]. Assuming the following "pre-equilibrium" model [33] $E + \mathrm{BrP} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} E\mathrm{BrP} \xrightarrow{k_2} E\mathrm{P}$ where BrPi is 3-bromopyruvate and $k_2 \ll k_{-1}$, it can be shown that, τ, the inactivation half-time, is equal to $\frac{\ln 2}{k_2} + \frac{K_{\mathrm{BrP}}}{(\mathrm{BrP})} \cdot \frac{\ln 2}{k_2}$ where $K_{\mathrm{BrP}} = k_{-1}/k_1$.

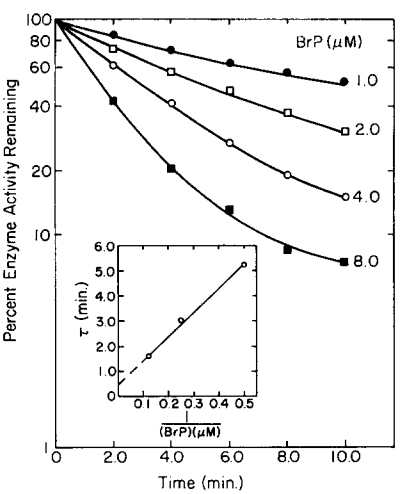

Fig. 5. Semilog plot of the time course of inactivation of *N. crassa* isocitrate lyase by 3-bromopyruvate (BrP) in 50 mM MOPS (sodium salt), 5.0 mM Mg^{2+}, 1.0 mM EDTA (pH 6.8). The procedure was as described in Materials and Methods. The inset shows inactivation half-time as a function of the reciprocal of the 3-bromopyruvate concentration.

When the measured inactivation half times are plotted against 1/(BrP) as shown in the inset, a straight-line plot is obtained. This is consistant with saturation kinetics as would be expected if 3-bromopyruvate were reversibly binding to a specific site prior to alkylation, presumably at or near the same site. K_{BrP}, as determined from the slope of the line, equals 20 μM. The limiting τ at saturating (BrP) equals 0.5 min.

The enzyme was found to be protected against inactivation by 3-bromopyruvate by its reactant, isocitrate, and its products, succinate and glyoxylate (see Table II). This protective effect suggests that the 3-bromopyruvate reacts with an amino acid residue at the active site of the enzyme.

TABLE II

PROTECTIVE EFFECT OF VARIOUS REACTANTS AGAINST INACTIVATION BY 3-BROMOPYRUVATE

The enzyme was treated with 5.0 μM 3-bromopyruvate for 6.0 min in the absence and presence of the reactants and products. Procedure was as described in Materials and Methods. The percent activities were measured relative to a control to which no 3-bromopyruvate was added.

Protective metabolite	Concentration (mM)	Percentage activity
None		24
Isocitrate	2.0	79
Glyoxylate	10	82
Succinate	10	59
Glyoxylate + succinate	10 + 10	100

DISCUSSION

Limited data suggest that isocitrate lyase is a cytoplasmic component in bacteria such as *P. indigofera* and *Hydrogenomonas facilis* [34, 35]. In numerous fatty seedlings, the enzyme is located with other enzymes of the glyoxylate cycle within microbodies termed glyoxysomes. These particles appear and subsequently disappear during seedling germination at a time of intensive conversion of fats to sucrose, a process probably essential to germination [36]. Thus glyoxysomes presumably evolved to fulfill a specific anaplerotic function and constituent enzymes may have arisen independently of prokaryotic counterparts during evolution. Particles analogous to glyoxysomes may exist in acetate-grown *N. crassa* although the evidence is incomplete [37]. The present comparison of *Pseudomonas* and *Neurospora* isocitrate lyase assumes special significance, then, because of the possibility that this enzyme is concentrated in microbodies of the latter source.

As indicated in the results, the subunit molecular weight of the isocitrate lyase from *N. crassa* is 67 000. This compares with subunit molecular weights of 48 000 and 123 000 for the enzymes from *P. indigofera* [16] and *Turbatrix aceti* [38], respectively. The *P. indigofera* enzyme has been shown to exist as a tetramer with a molecular weight of 206 000. The *T. aceti* enzyme also exists as a tetramer with a molecular weight of 480 000. The crude molecular weight as determined by gel filtration for

the native *N. crassa* isocitrate lyase was 270 000 or about 4 times the subunit molecular weight of 67 000. This strongly suggests that the *N. crassa* enzyme is also tetrameric.

During the purification step in which the *N. crassa* enzyme was run through a Sephadex G-200 column, the enzyme generally eluted at an elution volume that would be expected for a molecular weight of 270 000. However, on several occasions, it eluted at an elution volume which suggested a molecular weight of 130 000. This is appears that the *N. crassa* enzyme may also exist as a stable dimeric species.

In the companion paper to this one, several features of the active site of the *N. crassa* isocitrate lyase were inferred [11]. In this paper, the studies of the active site were extended. The C-terminal amino acid was found to be histidine, the penultimate amino acid was found to be phenylalanine and the enzyme was found to lose most if not all activity when the C-terminal amino acids were removed. In these important respects, the fungal enzyme is closely similar to isocitrate lyase from *P. indigofera* [16]. Our collective results suggest that the C-terminus has an important influence on the structure of the active site of isocitrate lyases or, alternatively, that it functions in catalysis.

3-Bromopyruvate was found to inactivate the *N. crassa* isocitrate lyase in a similar manner to that shown for the inactivation of the enzyme from *P. indigofera* [18]. With both enzymes isocitrate protects against the inactivation which shows saturation kinetics leading to the conclusion that bromopyruvate first binds to the enzyme in a rapid equilibrium and then more slowly inactivates the enzyme by alkylation of an amino acid residue at the active site. In the present studies, 3-bromopyruvate was found to bind to the *N. crassa* isocitrate lyase with a K_{BrP} of 20 μM at 30 °C and pH 6.8. In studies on the *P. indigofera* isocitrate lyase inactivated at pH 7.7 under similar conditions (100 mM MOPS, 5 mM Mg^{2+} and 1 mM EDTA), the K_{BrP} was found to be 30 μM. The 3-bromopyruvate was found to alkylate a cysteine residue in the active site of the *P. indigofera* isocitrate lyase [18]. Whether a cysteine is being alkylated in a similar position in the active site of the *N. crassa* enzyme remains to be seen. The 3-bromopyruvate binds to the *N. crassa* enzyme in one of three ways prior to alkylation: (i) at the glyoxylate portion of the active site, (ii) at both glyoxylate- and succinate-binding portions (by spanning) or (iii) at the succinate portion of the active site. With isocitrate lyase from *P. indigofera*, the third interpretation was favored because only the combination of glyoxylate plus succinate provided protection and the reaction mechanism was ordered, i.e. succinate binding required prior binding of glyoxylate.

In the present studies 10 mM glyoxylate alone provided good protection and 10 mM succinate provided some protection against inactivation. Because the reaction mechanism for the fungal enzyme inferentially becomes more random at higher concentrations of glyoxylate and succinate than that for the *Pseudomonas* enzyme [11], the protection patterns observed suggest that bromopyruvate spans the glyoxylate- and succinate-binding site. To recaptulate, then, the data obtained with 3-bromopyruvate for the *Pseudomonas* and fungal enzymes suggest similarities in topography for the active site. Nevertheless subtle differences are revealed when the protection patterns are compared.

The amino acid content of the isocitrate lyase from *N. crassa* is strikingly similar to the amino acid content for the isocitrate lyase from *P. indigofera*. The

value calculated by the $S\Delta Q$ function of Marchalonis and Weltman [32] is 22 suggesting that the proteins are homologous.

On the basis of the data presented in this paper and in the companion paper [11], the possibility that the isocitrate lyases from *N. crassa* and *P. indigofera* might have evolved from different origins cannot be completely eliminated. The differences in the pH optima and subunit molecular weights tend to suggest separate origins. Furthermore, it has not been possible to show any cross-reactivity by antibodies to either isocitrate lyase with the other isocitrate lyase (unpublished observations, J. O. Williams). However, in view of the apparently similar active sites, it seems more likely that the two isocitrate lyases have evolved from a common origin. Both have the same kinetic mechanism of action, and are regulated by the same metabolites with the same modes of inhibition [11]. Both require Mg^{2+} for activity, and also require protection from heavy metal ion poisoning for activity [11]. Both enzymes have C-terminal histidines with phenylalanine residues adjacent to them. The C-terminal amino acids are very important to, if not directly involved in, catalysis. 3-Bromopyruvate was found to inactivate both enzymes in similar manners. The inhibitions of both enzymes by small anions are nearly identical [11]. Thus it appears that the two isocitrate lyases have similar active-site structures which have been conserved in evolution. The close similarity of the amino acid contents suggests that the portions of the structures which were strongly conserved may be quite extensive. There has, however, been some divergence in the overall structures of the proteins as evidenced by the difference in the subunit molecular weights and the lack of immunological cross-reactivity. Overall, the weight of the evidence appears to favor the possibility that the two isocitrate lyases are homologous.

It will be of obvious interest to examine the properties of isocitrate lyase from a highly evolved plant species such as developing castor bean seedlings. In these seedlings, especially, the enzyme functions within cytoplasmic particles which have been well characterized ultrastructurally and which have a specific and well-defined biochemical function [36].

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REFERENCES

1 Kornberg, H. L. (1966) in Essays in Biochemistry (Campbell, P. N. and Greville, G. D., eds), pp. 1–31, Academic Press, New York
2 Tolbert, N. E. (1971) Annu. Rev. Plant Physiol. 22, 45–74
3 McFadden, B. A. (1969) Methods Enzymol. 13, 163–170
4 Spector, L. B. (1972) in The Enzymes (Boyer, P. D. ed.), pp. 357–389, Academic Press, New York
5 Olson, J. A. (1959) J. Biol. Chem. 234, 5–10
6 Smith, R. A. and Gunsalus, I. C. (1957) J. Biol. Chem. 229, 305–319
7 Kennedy, I. R. and Dilworth, M. J. (1963) Biochim. Biophys. Acta 67, 226–239

8 McFadden, B. A. and Howes, W. V. (1961) Biochim. Biophys. Acta 50, 179
9 Rao, G. R. and McFadden, B. A. (1965) Arch. Biochem. Biophys. 112, 294–303
10 McFadden, B. A., Rose, I. A. and Williams, J. O. (1972) Arch. Biochem. Biophys. 148, 84–88
11 Johanson, R. A., Hill, J. M. and McFadden, B. A. (1974) Biochim. Biophys. Acta 364, 327–340
12 Zanobini, A. and Firenzuoli, A. M. (1972) Physiol. Chem. Phys. 4, 166–172
13 Ozaki, H. and Shiio, I. (1968) J. Biochem. Tokyo 64, 355–363
14 Kornberg, H. L. (1965) in Regulations chez les Microorganismes, pp. 193–208, Centre National de la Recherche Scientifique, Paris
15 John, P. C. L. and Syrett, P. J. (1968) Biochem. J. 110, 481–484
16 McFadden, B. A., Rao, G. R., Cohen, A. L. and Roche, T. E. (1968) Biochemistry 7, 3574–3582
17 Shiio, I., Shiio, T. and McFadden, B. A. (1965) Biochim. Biophys. Acta 96, 114–122
18 Roche, T. E., McFadden, B. A. and Williams, J. O. (1971) Arch. Biochem. Biophys. 147, 192–200
19 John, P. C. L. and Syrett, P. J. (1967) Biochem. J. 105, 409–416
20 Harrop, L. C. and Kornberg, H. L. (1966) Proc. R. Soc. B. 166, 11–29
21 Andrews, P. (1965) Biochem. J. 96, 595–606
22 Kanarek, L. and Hill, R. L. (1964) J. Biol. Chem. 239, 4204–4206
23 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412
24 Moore, S. and Stein, W. H. (1963) Methods Enzymol. 6, 819–831
25 Hirs, C. H. W. (1956) J. Biol. Chem. 219, 611–619
26 Goodwin, T. W. and Morton, R. A. (1946) Biochem. J. 40, 628–632
27 Ambler, R. P. (1972) Methods Enzymol. 25B, 143–154
28 Schroeder, W. A. (1972) Methods Enzymol. 25B, 138–143
29 Korenman, S. G., Craven, G. R. and Anfinson, C. B. (1966) Biochim. Biophys. Acta 124, 160–165
30 Roche, T. E., Williams, J. O. and McFadden, B. A. (1970) Biochim. Biophys. Acta 206, 193–195
31 Siegel, L. M. and Monty, K. J. (1966) Biochim. Biophys. Acta 112, 346–362
32 Marchalonis, J. J. and Weltman, J. K. (1971) Comp. Biochem. Physiol. 38B, 609–625
33 Baker, B. R. (1967) in Design of Active-Site-Directed Irreversible Enzyme Inhibitors, p. 122, Wiley, New York
34 McFadden, B. A. and Howes, W. V. (1961) Biochim. Biophys. Acta 50, 179–181
35 McFadden, B. A. and Howes, W. V. (1962) J. Biol. Chem. 237, 1410–1412
36 Beevers, H. (1969) Ann. N.Y. Acad. Sci. 168, 313–324
37 Kobr, M. J., Vanderhaeghe, F. and Combepine, G. (1969) Biochem. Biophys. Res. Commun. 37, 640–645
38 Reiss, V. and Rothstein, M. (1974) Biochemistry 13, 1796–1800