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Isocitrate Lyase from Flax[1]

TERMINAL RESIDUES, COMPOSITION, ACTIVE SITE, AND CATALYSIS

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ABSTRACT

The cleavage of D_s-isocitrate catalyzed by isocitrate lyase from *Linum usitatissimum* results in the ordered release of succinate and glyoxylate. The glyoxylate analog 3-bromopyruvate irreversibly inactivates the flax enzyme in a process exhibiting saturation kinetics and protection by glyoxylate or isocitrate or the competitive inhibitor L-tartrate. Succinate provides considerably less protection. Results with 3-bromopyruvate suggest that this reagent modifies plant and prokaryotic isocitrate lyases differently. Treatment of the tetrameric 264,000-dalton flax enzyme with carboxypeptidase A results in a release of one histidine/subunit which is concordant with loss of activity. The only *N*-terminal residue is methionine. Treatment of flax enzyme with diethylpyrocarbonate at pH 6.5 selectively modifies two histidines per 67,000-dalton subunit. The reaction of one histidine residue is abolished by the binding of L-tartrate and the modification of one is coincident with inactivation. The carboxy-terminal and active-site modifications establish that one histidine residue/monomer is essential in the flax enzyme and considerably extend information heretofore available only for fungal and bacterial isocitrate lyase.

Isocitrate lyase (EC 4.1.3.1) is a major catalyst in the anaplerotic glyoxylate cycle. This cycle is operative in a wide range of microorganisms (30), germinating fatty seeds (3), and a variety of nematodes (2, 10, 22). In spite of its central importance in seedling metabolism and development, little is known about catalysis by plant isocitrate lyase.

The first isolation of isocitrate lyase from plants was described by Khan *et al.* (23). Several molecular properties of the enzyme from flax were characterized but data were insufficient to deduce the kinetic mechanism. Moreover, nothing was reported about the structure of the active site. Information about these characteristics of the plant enzyme has remained sketchy. Such information assumes special significance because plant isocitrate lyase functions within cytoplasmic organelles called glyoxysomes (3) whereas the prokaryotic enzyme is operationally 'soluble' (27, 28).

We now describe several catalytic properties of flax isocitrate lyase as well as the composition of this enzyme and data implicating an active-site histidine.

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MATERIALS AND METHODS

Materials. Mops,[4] trisodium DL-isocitrate, DTT, sodium glyoxylate monohydrate, isocitric dehydrogenase, monosodium $NADP^+$, phosphoenolpyruvate, itaconic acid, and DEP were purchased from Sigma. Succinic acid was obtained from Mallinckrodt. 3-Bromopyruvate and diisopropyl fluorophosphate-treated carboxypeptidase-A were the products of Calbiochem and Worthington, respectively. All more common chemicals were of high purity.

Isocitrate lyase was purified from *Linum usitatissimum* as described earlier (23). Protein was determined by the method of Lowry *et al.* (25) using BSA as a standard.

Enzyme Assays. Isocitrate lyase was assayed by the procedure of Roche *et al.* (36) with a slight modification. In a total volume of 1.0 ml, the incubation mixture (pH 7.5) contained: 100 μmol Mops, 5 μmol $MgCl_2$, 2 μmol DTT, 10 μmol trisodium DL-isocitrate, and enzyme. After 10 min incubation at 30°C, the reaction was quenched by addition of 0.1 ml of 1 M oxalic acid. A suitable aliquot was diluted to 2.6 ml with distilled H_2O followed by the addition of 0.1 ml of 1% (w/v) phenylhydrazine-hydrochloride. After thorough mixing, the tubes were chilled in ice for 10 min, and 1.5 ml prechilled concentrated HCl added with rapid mixing followed after 5 min by addition of 0.1 ml of 5% (w/v) potassium ferricyanide. After thorough mixing and incubation for 15 min at 25°C, the color intensity was read at 520 nm. Reaction rates were proportional to enzyme and the rate was constant over a 25-min incubation period.

To examine the inhibition of isocitrate lyase by glyoxylate, the reaction rate was determined by assaying succinate using succinic dehydrogenase (38).

The condensation reaction catalyzed by isocitrate lyase was studied as decribed by Johanson *et al.* (19) by coupling with an excess of $NADP^+$-isocitrate dehydrogenase.

Amino Acid Analysis. Isocitrate lyase was exhaustively dialyzed against 0.1 M NaCl prior to amino acid analysis. Aliquots of the dialyzed enzyme were transferred to hydrolysis vials having norleucine as an internal standard and the contents lyophilized. After addition of 0.5 ml of 6 N HCl, the vials were degassed *in vacuo* and sealed under N_2. The hydrolysis was carried out at 110°C and all hydrolysates were analyzed with a Beckman 120C automatic amino acid analyzer by the method of Moore and Stein (33). Half-cystine was determined as cysteic acid after performic acid oxidation by the method of Hirs (18).

***N*-Terminal Analysis.** Protein (1 mg) was dansylated in 60 mM phosphate buffer (pH 8.5) containing 4 M urea at room temperature (17). The protein was precipitated with 10% TCA and the precipitate recovered by centrifugation, and washed twice with 1

[4] Abbreviations: Mops, morpholinopropane sulfonic acid; DEP, diethylpyrocarbonate.

M HCl. The dansyl enzyme was hydrolyzed, the hydrolysate was taken to dryness, and the residue suspended in 0.1 ml of a mixture of acetone-1 N HCl (9:1, v/v). TLC was used for identification of dansylamino acids using solvent system A of Morse and Horecker (34) and solvent system B of Deyl and Rosmus (12). Migrations were compared with standard dansylamino acids (Sigma) as necessary.

Studies with Carboxypeptidase A. Isocitrate lyase from flax was treated with carboxypeptidase A as described by Ambler (1). Diisopropyl fluorophosphate-treated carboxypeptidase A (1 mg), which was free of chymotrypsin and trypsin activities, was rinsed with 50 mM Mops buffer (pH 8.0) containing 5 mM Mg^{2+} and 1 mM EDTA. After centrifugation, the crystals were dissolved in 5 ml of the same buffer containing 10% NaCl. Stocks of isocitrate lyase (1 mg/ml) were prepared in the above buffer containing 1 mM DTT but lacking 10% NaCl.

Zero-time analysis was afforded by adding 0.5 ml of stock isocitrate lyase and 0.15 ml of carboxypeptidase A stock directly to 0.65 ml of 10% TCA. Further studies were done by adding 0.75 ml of the stock carboxypeptidase to 2.5 ml of the isocitrate lyase stock and the mixture was incubated at 30°C. At intervals, samples were withdrawn and added to an equal volume of 10% TCA. Simultaneously, samples were withdrawn to assay isocitrate lyase activity in the presence of a final contentration of 0.75 mM β-phenylpropionate to quench carboxypeptidase A action. The samples treated with TCA were centrifuged and the pellets rinsed with 5% TCA. The supernatants were combined, acidified further with 6 M HCl, and extracted twice with diethylether. The extracted supernatants were dried *in vacuo* over NaOH and taken up in citrate buffer to determine the amino acid content using a Beckman Spinco 120C automatic amino acid analyzer.

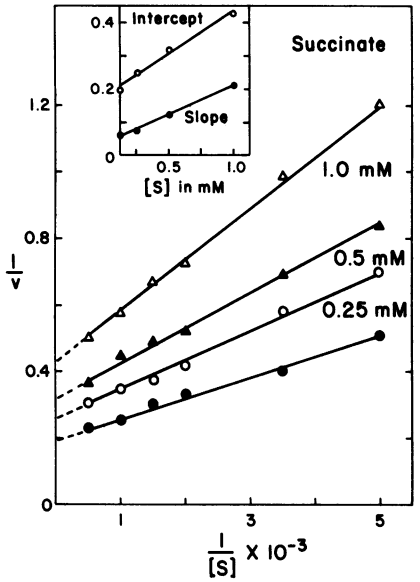

FIG. 1. Lineweaver-Burk plot showing the inhibition of isocitrate cleavage (in μmol/min) by succinate (in molar units). The inset shows replots of the slopes (●) and intercepts (○).

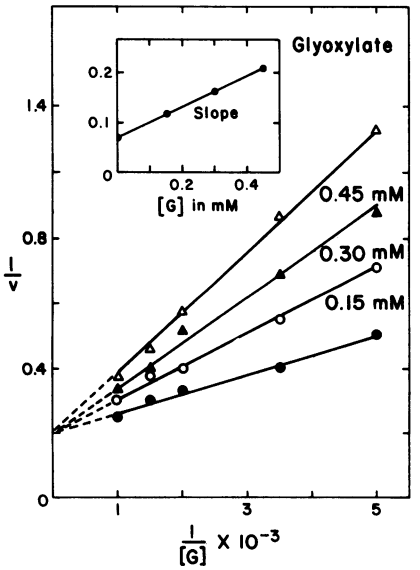

FIG. 2. Lineweaver-Burk plot showing the inhbition of isocitrate cleavage (in μmol/min) by glyoxylate (in molar units). The inset shows a replot of the slopes.

Hydrazinolysis. The method of Schroeder (41) was followed to prepare hydrazine and to carry out hydrazinolysis. To remove hydrazine and to separate free amino acids from hydrazides, the procedure of Korenman *et al.* (24) was used.

Studies of Inactivation by 3-Bromopyruvate. The reducing agent DTT, present in enzyme, was removed by gel filtration using a Sephadex G-50 column equilibrated with 50 mM Mops, 5 mM Mg^{2+}, and 1 mM EDTA (pH 7.5). An appropriate amount of 3-bromopyruvate was added to give the desired concentration. The reaction was stopped at different time intervals by the addition of DTT to a final concentration of 5 mM. Isocitrate (2 mM) was added after 10 min and isocitrate lyase activity was determined as described. In studies of protection of the enzyme by substrate and products, the test compounds were removed by filtration on

Table I. *Amino Acid Composition of Flax Isocitrate Lyase*

Amino Acid	Extrapolated or Maximum Value	Nearest Integral No. of Amino Acid Residues per 264,000-D Molecule of Protein[a]	Composition of Native Enzyme from[b]		
			Linum usitatissimum	*P. indigofera*[c]	*N. crassa*[d]
	nmol				
Asp	47.7	193	2.22	2.77	3.51
Thr[e]	28.4	115	1.32	1.69	1.83
Ser[e]	22.4	91	1.04	1.26	1.71
Glu	55.9	226	2.59	3.38	4.28
Pro	13.3	54	0.62	1.12	1.33
Gly	45.7	185	2.13	2.47	2.24
Ala	62.4	253	2.91	3.74	4.23
Val[f]	46.8	190	2.18	2.19	1.86
Ile[f]	23.0	93	1.07	1.16	1.80
Leu	34.1	138	1.58	1.97	2.81
Tyr	19.2	77	0.88	1.18	1.15
Phe	21.6	87	(1.00)	(1.00)	(1.00)
Lys	30.6	124	1.42	1.60	2.75
His	12.7	51	0.58	0.66	0.63
Arg	25.0	101	1.16	1.32	1.61
Cys[g]	5.9	24	0.27	0.29	0.47
Met[g]	13.0	53	0.61	0.60	0.83
Trp	6.7	27	0.31	0.29	0.75

[a] Values were corrected from 95% recovery based upon the use of norleucine as an internal standard to 100% recovery. The amount of protein analyzed was 60 μg for each hydrolysis time of 24, 48, and 72 h.

[b] Composition normalized with respect to mole content of phenylalanine.

[c] Data obtained from Shiio *et al.* (24)

[d] Data obtained from Johanson *et al.* (20).

[e] Residues estimated from extrapolation to zero hydrolysis time.

[f] Residues estimated from the 48-h value.

[g] Residues estimated after performic acid oxidation.

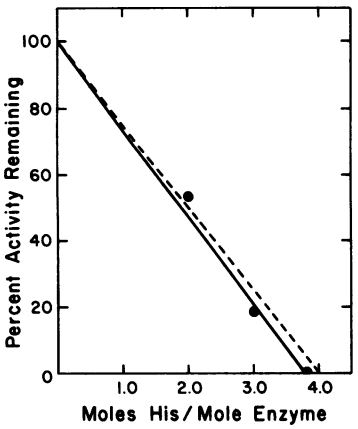

FIG. 3. Loss of isocitrate lyase activity with carboxypeptidase A-catalyzed removal of histidine. The dashed line (- - -) shows the theoretical decay assuming a four-subunit model and that each subunit behaves as an independent catalytic entity.

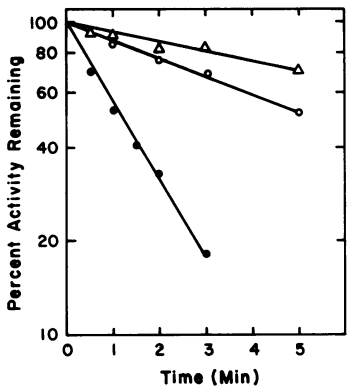

FIG. 4. Effect of substrate and competitive inhibitor on the rate of inactivation of isocitrate lyase by 1.0 mM DEP. Experiments were conducted with DEP in the absence (●) or presence (△) of 2.5 mM Ds-isocitrate, or 5 mM sodium malonate (○).

Table II. *Protection against Inactivation by 3-Bromopyruvate*

The enzyme was treated with 5 μM 3-bromopyruvate (BrP) for 5 min with and without addition of protective ligands. Methodology was as described in "Materials and Methods." The remaining activity was compared with a control lacking BrP but having the same amount of test ligand.

Additions	Concn. of Test Ligand	Activity Remaining
	mM	%
Bromopyruvate (BrP)		30
Isocitrate + BrP	2.5	85
L-Tartrate + BrP	10	78
Succinate + BrP	10	51
Glyoxylate + BrP	10	89
Glyoxylate + Succinate + BrP	10 + 10	97

Table III. *Protection against Modification of Flax Isocitrate Lyase with DEP*

Isocitrate lyase in 100 mM phosphate, pH 6.5, was incubated at 30°C with protective ligand 5 min prior to the addition of DEP at a final concentration of 0.7 mM. At various times, the reaction was quenched and enzyme activity determined. The remaining activity was compared with a control lacking DEP but having the same amount of protective agent and ethanol.

Additions	Protective Ligand	Activity Remaining after Incubation for		
		1 min	3 min	5 min
	mM	%		
DEP		52	36	7
L-Tartrate + DEP	10	93	74	57
Succinate + DEP	5	74	63	35
P-enolpyruvate + DEP	5	71	59	21
Glyoxylate + DEP	5	87	72	49

Sephadex G-50 before enzyme activity was assayed.

Modification by DEP. To enzyme solution (0.5–1.5 mg/ml) in 100 mM sodium phosphate (pH 6.5) a freshly prepared solution of DEP in absolute ethanol was added to the final concentrations indicated in the figures and tables. In no case did the final concentration of ethanol exceed 2%; the controls contained the same volume of ethanol. There was no effect of ethanol on enzyme activity during the course of the study. During incubation at 30°C aliquots were removed at intervals and remaining enzyme activity was assayed as described earlier. Modification by DEP was quenched by the addition of histidine to a final concentration of 20 mM. The UV spectrum was recorded to measure the change at 240 nm due to carboethoxylation of the histidine residues using the Cary spectrophotometer (model 14). The number of histidines modified was determined from absorption at 240 nm using a molar extinction coefficient of 3200 M^{-1} cm^{-1} (32).

After removal of DTT by gel filtration, the free sulfhydryl content of isocitrate lyase was determined (13) before and after treatment with DEP using 5,5'-dithiobis(2-nitrobenzoic acid) in the presence of SDS. Tyrosine and tryptophan were estimated analogously but in the absence of SDS by the method of Goodwin and Morton (16).

Reactivation of DEP-Treated Enzyme. Reaction of DEP-treated enzyme with hydroxylamine was carried out at pH 7.0 at a final concentration of 0.4 M in 100 mM Tris-HCl buffer. The decrease in A at 240 nm was recorded and aliquots removed at intervals to measure enzyme activity. Hydroxylamine was removed prior to enzyme assay by passing the reactivated enzyme through a Sephadex G-25 column (PD-10, Pharmacia).

For protection studies, enzyme was preincubated for 5 min with protective ligands at the concentrations specified prior to the addition of DEP.

RESULTS

Condensation Reaction of Isocitrate Lyase. The condensation reaction (at pH 7.5) was performed at several fixed concentrations of one substrate while at each fixed concentration, the concentration of the second substrate was varied. Intercepts of double reciprocal plots of 1/v *versus* 1/[succinate] at various concentrations of glyoxylate, G, were linear with respect to 1/[G] and the converse was also true (data not shown). Such a response is compatible with either ordered binding of substrates or a rapid equilibrium random mechanism (8, 44).

Enzyme Inhibition by Products. Both products of isocitrate cleavage were found to be strong inhibitors of isocitrate lyase (Figs. 1 and 2). As shown in Figure 1, succinate was a linear noncompetitive inhibitor in the terminology of Cleland (8), with a K_i of 0.7 mM determined from the slope. In contrast, glyoxylate was a linear competitive inhibitor of the enzyme (Fig. 2) with a K_i value of 0.22 mM. Both K_i values were measured at 30°C, pH 7.5.

Kinetic Mechanism. On the basis of the influence of both substrates on the rate of the condensation reaction and in terms of product inhibition patterns (Figs. 1 and 2), we conclude that the

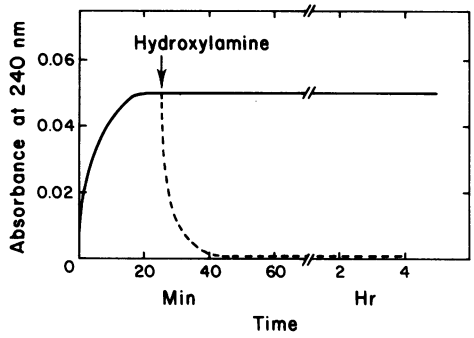

FIG. 5. Reaction of DEP with flax isocitrate lyase. The enzyme (0.5 mg/ml) in 100 mM phosphate, pH 6.5, was incubated with 1.0 mM DEP. The differential absorption spectrum was taken at 240 nm. An arrow shows the addition of hydroxylamine to 0.4 M.

Table IV. *Reactivation DEP-Modified Isocitrate Lyase by Hydroxylamine*

Isocitrate lyase was inactivated with DEP and the reaction quenched with 20 mM histidine before the addition of 0.2 M hydroxylamine in Tris-HCl buffer, pH 7.0. After the indicated incubation period, the enzyme was gel filtered into the same buffer and assayed for activity.

Concn. DEP	Residual Activity of the Modified Enzyme			
	After 5 min incubation at 30°C	After 2 h incubation at 4°C	After further incubation with hydroxylamine for	
			2 h at 25°C	12 h at 4°C
mM			%	
0.20	45	49	70	89
0.40	36	33	63	82

kinetic mechanism is ordered. In an ordered uni-bi reaction, the product bound by free enzyme (E) is a linear competitive inhibitor with respect to reactant isocitrate (Isocit) as was the case for glyoxylate, G (8, 9). The second product, succinate, was a linear noncompetitive inhibitor of isocitrate cleavage as would be expected if it was bound to the EG form (8, 9). We infer the following binding mechanism:

$$E + Isocit \rightleftharpoons^{EIsocit}_{EGS} \rightleftharpoons S + EG \rightleftharpoons E + G$$

Inhibition by Substrate Analogs. Phospoenolpyruvate was a linear noncompetitive inhibitor of isocitrate cleavage in the concentration range of 0.1 to 0.5 mM. A K_i of 0.13 mM (at pH 7.5, 30°C) was found from the slopes.

Kinetic analysis showed that itaconate was also a linear noncompetitive inhibitor with a K_i of 20 μM at pH 7.5, 30°C.

Amino Acid Analysis. The amino acid composition of flax isocitrate lyase is shown in Table I. Amino acid compositions of isocitrate lyase from *P. indigofera* and *N. crassa* are also presented for comparison. SΔn values as described by Cornish-Bowden (11) have been calculated on the basis of all residues. The values are 9,584 and 1,271 for comparisons with the fungal and pseudomonad enzyme, respectively. These values suggest that amino acid sequence resemblances between flax, and either fungal or pseudomonad isocitrate lyases will prove to be insignificant (11).

The SΔn analysis is very effective in correlating amino acid compositions with sequence homologies when test proteins are of similar chain length. On the other hand, when test proteins are of appreciably different length such as is the case for the flax and pseudomonad enzymes (mol wt = 67,000 and 51,000, respectively), the SΔQ analysis of Marchelonis and Weltman (26) enables a more valid test for sequence homology. This parameter, SΔQ, equals $S(X_{i,j} - X_{k,j})^2$ where X_j is the content of a given amino acid of type j (excluding half-cystine and tryptophan) expressed as mole percentage, and the subscripts i and k identify the particular proteins which are being compared. Marchelonis and Weltman (26) compared over 5,000 pairs of proteins, most of known sequence, and found that for 98% of unrelated proteins SΔQ was >100. In no case was SΔQ <50 found for unrelated proteins. The values are 34 and 7 for the comparison of flax isocitrate lyase with those from *N. crassa* and *P. indigofera*, respectively. Thus, the pseudomonad and flax enzymes are remarkably similar and may prove to be extensively homologous.

The partial specific volume calculated from amino acid analysis was 0.723 ml/g for the flax enzyme.

The Amino Terminus. The *N*-terminal amino acid residue of isocitrate lyase was found to be methionine when TLC was done. Although dansylalanine and dansylphenylalanine had similar mobilities, chromatography of dansylphenylalanine, dansylalanine, and dansylmethionine in solvent system A (34) or B (12) gave unambiguous separations. TLC of hydrolyzed dansylenzyme using the solvent systems mentioned yielded one spot corresponding to dansylmethionine. Only one spot was obtained when the sample was co-chromatographed with authentic dansylmethionine (Sigma) added as an internal standard. This indicated methionine as the only *N*-terminal residue.

The Carboxy Terminus. Data from digestion of isocitrate lyase with carboxypeptidase A or from hydrazinolysis revealed histidine as the *C*-terminal amino acid. No other amino acid residue was released during digestion with carboxypeptidase A. It was also found that the number of histidines released was 4/mol enzyme. Figure 3 shows the quantitative relationship between histidine removal and loss of isocitrate lyase activity. This indicates that the *C*-terminal histidine residues are essential for enzyme activity.

Inactivation with 3-Bromopyruvate. Flax isocitrate lyase was inactivated by 3-bromopyruvate. The inactivation exhibited saturation kinetics and the half-time was 0.6 min at pH 7.5, 30.0°C.

The enzyme was found to be protected against inactivation by its substrate as well as products (Table II). The protection study suggests involvement of one or more amino acids which is/are at or near the active site.

Inactivation of Isocitrate Lyase with DEP. The reaction of flax isocitrate lyase with DEP at pH 7.0 and 30°C resulted in rapid inactivation. The rate of inactivation was first-order with respect to DEP and yielded $t_{1/2}$ of 0.4 min at saturating DEP. Both substrate and products protected isocitrate lyase against inactivation with DEP (Fig. 4; Table III). The data in Table III show that the protection against DEP inactivation fell off with time which is consistent with other enzymic studies (32) and would be expected for protection by reversibly-bound substrates or analogs.

The Nature of Modification by DEP. Although DEP reacts with histidine residues relatively specifically in the pH range of 6 to 7 (32), other residues may be modified in neutral or slightly alkaline media (5) including cysteine and tyrosine residues (4, 15).

The reaction of proteins with DEP results in an increase in A at 240 nm if histidyl residues are modified and a decrease in A at 280 nm if tyrosyl residues are modified (32). The increase in differential absorption at 240 nm during the modification of isocitrate lyase with DEP indicated that histidyl residues were modified (Fig. 5). About 8.25 histidyl residues were modified with

Table V. *Determination of Tyrosine, Tryptophan, and Cysteine in Native and DEP-Modified Enzyme*

Isocitrate lyase was inactivated with 1 mM DEP for 10 min. Amino acid residues were determined as described in the text.

Enzyme	Activity	Tyrosine	Tryptophan	Cysteine
	%	*residues/molecule*		
Native	100	76.7	27.3	7.9
Modified	5	76.2	28.6	8.2

DEP per 264,000-D isocitrate lyase.

Treatment of DEP-inactivated enzyme with hydroxylamine reulted in restoration of activity (Table IV). Incubation without hydroxylamine did not restore activity. Since hydroxylamine cleaves the ethoxyformyl-histidyl and -tyrosyl bonds but does not cleave the more stable ethoxyformyl-lysyl bond (31, 32); the inactivation of isocitrate lyase by DEP appears to be due to the modification of histidyl and/or tyrosyl residues. Also, it is clear from Figure 5 that the differential absorption at 240 nm of DEP-treated enzyme disappeared during hydroxylamine treatment.

The specific modification of histidyl residue(s) with DEP is supported by the observation that the numbers of sulfhydryl groups, and tyrosine and tryptophan residues were the same in native and modified enzyme (Table V). From the thiol content and cysteic acid analysis (Table I), it can be estimated that there are two cysteine and two cystine residues per subunit of the flax enzyme.

Stoichiometry of the Reaction. The loss of isocitrate lyase activity plotted against the number of histidines modified (calculated from absorption at 240 nm) was linear early in the course of inactivation but deviated from the linearity when the degree of inactivation exceeded 50% (Fig. 6). When the linear portion of the graph was extrapolated to zero activity, a stoichiometry of one essential histidine modified per subunit was obtained.

The stoichiometry of essential histidine modification was also investigated spectrophotometrically (at 240 nm) with the known competitive inhibitor, L-tartrate. When isocitrate lyase was first incubated with this compound for 5 min and then modified by DEP in the presence or absence of L-tartrate, only 1.2 histidine residues were modified with DEP in the presence of L-tartrate (Fig. 7, curve B). In contrast, 2.2 residues were modified in the absence of tartrate (Fig. 7, curve A). The difference between the data obtained in the presence and absence of this competitive inhibitor (Fig. 7, curve C) suggests that the modification of one histidine residue is prevented by the binding of a substrate analog.

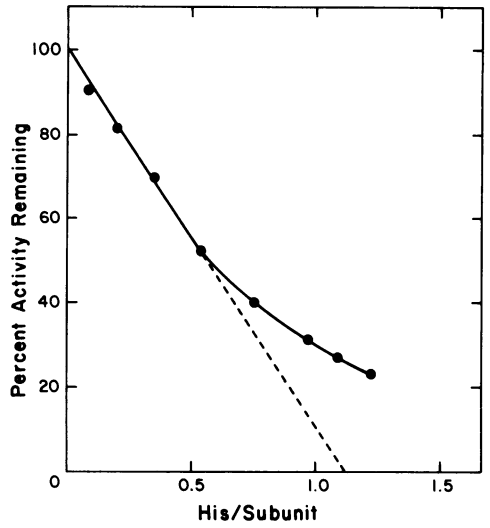

FIG. 6. Stoichiometry of inactivation of isocitrate lyase by DEP. The enzyme sample was inactivated with 0.5 mM DEP. Aliquots were removed at intervals and assayed for enzyme activity. The number of histidines modified was calculated from the changes in absorbance at 240 nm.

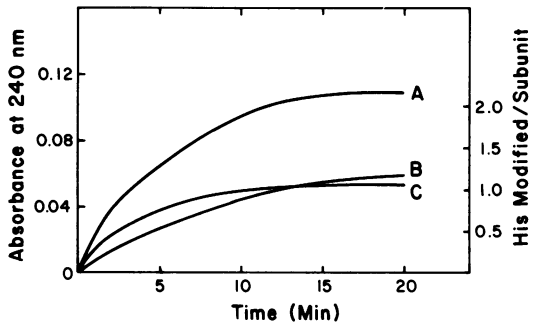

FIG. 7. The differential absorption at 240 nm of isocitrate lyase inactivated by DEP in the absence (A) and presence (B) of L-tartrate. The enzyme was preincubated for 5 min at 30°C with 10 mM L-tartrate before addition of DEP to 0.5 mM. The change in A at 240 nm was used to calculate the number of histidines modified. Curve C was generated by subtracting curve B from A.

DISCUSSION

Although isocitrate lyases from several sources have been purified and studied, little was known about the plant enzyme prior to the present investigation. In bacteria such as *P. indigofera* (27) and *Hydrogenomonas facilis* (28), isocitrate lyase is a cytoplasmic component, whereas in the fatty seedlings the enzyme is located within microbodies termed glyoxysomes (3). Thus, comparisons of the enzyme by istelf are not only of evolutionary interest but raise questions about the relationship between structure, function, and compartmentation. In the face of these considerations, it is interesting that isocitrate lyases from bacteria, plants, and fungi are so similar. Only in size do they differ appreciably. The bacterial enzymes are 206,000 and 180,000 daltons from *Pseudomonas indigofera* and a thermophilic *Bacillus* sp., respectively (6, 29), and the green algal enzyme from *Chlorella pyranoidosa* has a mol wt of 180,000 (21), a value similar to that reported for lupine isocitrate lyase (43). In contrast, enzymes from *Neurospora crassa*, flax, and cucumber have mol wt of 270,000 (20), 264,000 (23), and 255,000 (14), respectively. In all cases investigated, the enzymes are tetrameric (7).

A quantitative comparison of composition suggests that the pseudomonad, fungal, and flax enzymes may have extensive regions of homology, yet subunits from the latter two sources (64,000–67,000 D) are about 33% larger than that of the bacterial enzyme (7). It is of interest, then, that in both pseudomonad (29) and flax enzymes, methionine and histidine are *N*- and *C*-terminal, respectively. In the fungal enzyme, histidine is also *C*-terminal and the *N*-terminus is unknown (20). In both fungal and pseudomonad enzymes, the penultimate residue at the carboxy-terminus is phenylalanine (20, 29), yet this is not the case with the flax enzyme.

With regard to catalysis by isocitrate lyase, the fungal (19), pseudomonad (44) and flax enzymes sequentially release succinate and glyoxylate in the cleavage reaction and the kinetic mechanism is preferentially ordered uni-bi. This is in accord with the observed product inhibition patterns. The effects of inhibitors of the flax enzyme like itaconate and P-enolpyruvate, both succinate analogs, are more complex. For an ordered uni-bi reaction in which a dead-end inhibitor (I) interacts as follows:

$$E \underset{k_{-1}}{\overset{(G)k_1}{\rightleftharpoons}} EG \underset{k_{-2}}{\overset{(S)k_2}{\rightleftharpoons}} EGS \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EIsocit \underset{k_{-4}(I)}{\overset{k_4}{\rightleftharpoons}} E + Isocit$$

$$I \Downarrow K_i$$

$$EGI$$

the reciprocal of the initial velocity equation for isocitrate (Isocit) cleavage when glyoxylate (G) and succinate (S) concentrations are zero is (8):

$$\frac{1}{v} = \frac{1}{V_r}\left[1 + \frac{[I]}{K_i}\frac{k_{-2}k_{-3}}{k_{-2}k_{-3} + k_{-1}k_3 + k_{-1}k_{-3} + k_{-1}k_{-2}} + \frac{K_{Isocit}}{[Isocit]}\right]$$

Thus, a deadend inhibitor which interacts only as shown should be a linear uncompetitive inhibitor, *i.e.*, show only an intercept effect in Lineweaver-Burk plots. In the present work, both slope

and intercept effects were observed with itaconate and P-enolpyruvate. One interpretation is that itaconate and P-enolpyruvate can also interact with free enzyme. Regardless of the exact mechanism of action, both of these ligands are strong inhibitors. For example, the K_i for itaconate, the most potent inhibitor of isocitrate lyase known, is 20 μM at pH 7.5 (30°C) for the flax enzyme in comparison with 7 μM at pH 7.7 (30°C) for the pseudomonad enzyme (44).

Studies of the irreversible inactivation of flax isocitrate lyase by 3-bromopyruvate establish that saturation kinetics obtain and that isocitrate or glyoxylate protects against inactivation. L-tartrate, a linear competitive inhibitor with respect to isocitrate, also protects but succinate gives little protection. The data suggest that the alkylating agent bromopyruvate modifies a portion of the active center required for action on glyoxylate, but the possibility that the bound reagent also spans the succinate-binding moiety cannot be excluded because of the ordered kinetic mechanism. In this respect, the flax enzyme is closely similar to the fungal enzyme (20) but considerably different from the pseudomonad enzyme in which the succinate site is modified by the reagent (35, 37).

Of interest is the observation that flax isocitrate lyase, like that from *Neurospora* (20) and *Pseudomonas* (29), is rapidly inactivated by removal of *C*-terminal histidine. In all three cases, the decay in activity is in exact concordance with *C*-terminal histidine release suggesting that each subunit behaves as an independent catalytic entity. The presence of *C*-terminal histidine is either required for catalysis or structural maintenance. A possibly related observation is that the flax isocitrate lyase is inactivated when one histidine per subunit is modified by DEP. Additional slower modification of a second nonessential histidine occurs. L-Tartrate provides some protection against modification of the essential histidine and isocitrate or malonate, a linear competitive inhibitor, protects against inactivation. These observations suggest that a histidine residue is in the active center. That it functions in catalysis is reasonable but unproven. With both fungal (40) and pseudomonad enzymes (39), a slightly acidic residue (pKa 6.1–6.9) functions in catalysis. This residue is plausibly histidine.

To recapitulate, most known structural and catalytic properties of isocitrate lyase have been conserved during evolution. Perhaps the most striking is the presence of an essential *C*-terminal histidine residue. Whether this is also the active-site histidine in flax isocitrate lyase remains to be established.

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