Isocitrate Lyase from *Pseudomonas indigofera*. V. Subunits and Terminal Residues and the Relation to Catalytic Activity*

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ABSTRACT: Isocitrate lyase from *Pseudomonas indigofera* is composed of four subunits. Treatment with carboxypeptidase A results in the rapid release of histidine that approaches a tentative limiting yield of 4 moles/mole of isocitrate lyase. Hydrazinolysis confirms that histidine is carboxy terminal. Phenylalanine may be penultimate. Histidine removal catalyzed by carboxypeptidase A shows excellent concordance with loss in activity of isocitrate lyase. The sedimentation coefficient of so-altered isocitrate lyase is identical with that of native enzyme. Isocitrate lyase is resistant to proteolysis by carboxypeptidase B and both native and urea-treated enzyme are resistant to leucine aminopeptidase. Studies with 1-dimethylaminonaphthalene-5-sulfonyl chloride indicate that methionine is the amino-terminal residue. Isocitrate lyase is irreversibly inactivated by 4.2 mM 2-bromomalonate, an analog of the competitive inhibitor, malonate. Protection against this inactivation is provided by the substrate, isocitrate, at 2.2 mM.

The pH dependence of inhibition by 2-bromomalonate suggests involvement of nucleophilic groups of an average pK_a of 6.8 on isocitrate lyase. Guanidine hydrochloride at 0.8 M rapidly inhibits isocitrate lyase to the extent of 88% but this is not accompanied by spectral alteration or dissociation of the enzyme. Removal of guanidine hydrochloride through dialysis largely reverses the inhibition. Onset of inhibition by 2.4 M urea is slower and the inhibition is less extensive. Inhibition by 3 M urea is not reversed by dialysis. Dissociation of isocitrate lyase by 3–6 M guanidine hydrochloride has been studied with the meniscus-depletion ultracentrifugal technique of Yphantis. The subunits have an average molecular weight (uncorrected for preferential interaction of third component) of 48,200. The molecular weight of native enzyme is about 206,000. The physical and chemical data suggest that isocitrate lyase is comprised of four subunits of similar, if not identical, size. This number of subunits has been supported directly with electron microscopy.

Isocitrate lyase (D_s-isocitrate glyoxylate-lyase, EC 4.1.3.1) is a major catalyst in the important anaplerotic sequence the glyoxylate cycle. This reaction sequence is fairly widely distributed in nature and generally plays a key role in microbial growth on monocarboxylic acids and in germination of fatty seedlings (Kornberg, 1966; Beevers, 1961). In *Escherichia coli*, metabolite flow through the glyoxylate cycle seems to be regulated by the concentration of phosphoenolpyruvate which may diminish the rate of formation and activity of isocitrate lyase (Kornberg, 1966). In pseudomonads, the activity of this key catalyst is primarily inhibited by certain Krebs cycle intermediates, a phenomenon that may contribute to control of isocitrate utilization by the competing oxidative and biosynthetic cycles (McFadden and Howes, 1963; Rao and McFadden, 1965). In spite of the relevance to function of the glyoxylate cycle, there is as yet little information about the subunits or other structural features of isocitrate lyase. These aspects are the subject of the present communication.

Materials and Methods

Isocitrate Lyase. The enzyme was prepared from extracts of *Pseudomonas indigofera* M1 (Shiio *et al.*, 1965a). The final product was recovered from the pooled eluents from chromatography on DEAE-cellulose by reprecipitation from alkaline ammonium sulfate between 0.47 and 0.53 saturation. The product is known to contain less than 1% minor components by the criterion of electrophoresis in polyacrylamide gel and appeared to be homogeneous in sedimentation and diffusion studies (Shiio *et al.*, 1965a). In the present studies, the enzyme had a specific activity in the range of 28–31 and gave a single band with electrophoresis on cellulose acetate or showed virtual homogeneity upon electrophoresis in polyacrylamide gel disks (at a protein concentration of 20 μg/ml).

Enzyme was stored at −20° at a concentration of at least 3 mg/ml as estimated from ultraviolet absorption at 280 and 260 mμ (Shiio *et al.*, 1965a). However the A_{280}/A_{260} ratio of the enzyme varied from 1.70 to 2.10, presumably as a function of the conformational state. This necessitated a more reliable estimate of enzyme con-

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centration. This was obtained after dialysis against pure H_2O by relating dry weight (Shiio *et al.*, 1965a) to absorbancy of isocitrate lyase itself after treatment with Folin–Ciocalteu reagent (Layne, 1957).

Activation and Assay of the Enzyme. The enzyme was activated with GSH and assayed as described earlier (Shiio *et al.*, 1965a; McFadden and Howes, 1960).

In studies using the inhibitor 2-bromomalonate (K & K Laboratories, Inc.), it was necessary to remove GSH after enzyme activation. This was done by gel filtration on Sephadex G-50 and the enzyme was subsequently assayed in the presence of EDTA as described earlier (Shiio *et al.*, 1965b).

Studies with Dansyl[1] *Chloride.* Dansylation reactions were carried out by the method of Gray (1967) described for proteins. Protein (1–6 mg) was reacted overnight in urea at room temperature. The precipitate recovered by centrifugation was resuspended in 0.5 ml of water and then treated with 1.0 ml of 20% (w/v) trichloroacetic acid. This was followed by five or more successive washes with a 1.0-ml solution of 95% ethanol containing 0.25 M HCl, and finally two washes with 1.0 ml of anhydrous ether. The protein derivative was hydrolyzed, the hydrolysate was taken to dryness, and the residue was extracted with three 0.1 ml-volumes of water-saturated ethyl acetate (Gray, 1967). Thin-layer chromatography was used for identification of dansylamino acids using solvent systems C and D of Morse and Horecker (1966) and solvent system B of Nedkov and Genov (1966). Using the latter system it was necessary to ensure saturation of the solvent with NH_3 by bubbling NH_3 into the mixture of ethyl acetate, isopropyl alcohol, and concentrated NH_3. Migrations were compared with standards (Pierce Chemical Co. and Calbiochem) as necessary.

Hydrazinolysis. The method of Braun and Schroeder (1967) was used for preparation of hydrazine and hydrazinolysis of 2.6 mg of isocitrate lyase. Hydrazine was removed and free amino acids were separated from hydrazides by the techniques of Korenman *et al.* (1966).

Studies with Carboxypeptidase. DFP-treated, chymotrypsin-free, trypsin-free carboxypeptidase A was obtained from Worthington Biochemical Corp. (COADFP 6FC) and 340 units (68 units/mg) diluted to 1 ml with cold 0.1 M potassium phosphate buffer (pH 7.6). The suspension was then exhaustively dialyzed at 2° against the same buffer, mixed to ensure a uniform suspension, and diluted to 20 units/ml with 0.1 M phosphate buffer containing 10% NaCl. A stock of isocitrate lyase (1 ml) (3 mg) was exhaustively dialyzed at 2° against 0.01 M potassium phosphate buffer containing 5 mM $MgCl_2$ (pH 7.66). After dialysis, the volume was adjusted with this buffer to 3.426 ml and aliquots were removed for activity and protein (Layne, 1957) assay. Two 0.36-ml portions were then each transferred to 0.13-ml aliquots of 10% trichloroacetic acid and 0.09 ml of the carboxypeptidase solution was added to each. The solutions were frozen at −80° and retained for amino acid analysis. To the remainder of the solution of isocitrate lyase at 30°, one-fourth volume of carboxypeptidase A (mass of isocitrate lyase–carboxypeptidase A, 10:1) was added. At desired times, small aliquots were removed and rapidly frozen for subsequent activity assay. Two 0.45-ml aliquots were also rapidly transferred to 0.13-ml portions of 10% trichloroacetic acid and frozen for subsequent amino acid analyses. In all cases aliquots for activity assay were transferred to 0.1 ml of 0.01 M β-phenylpropionate in 0.05 M Tris-HCl containing 3 mM $MgCl_2$, pH 7.7 (25°). They were then rapidly frozen and subsequently assayed in the usual way in the presence of a final concentration of 7.5×10^{-4} M β-phenyl propionate.

For studies with carboxypeptidase B, enzyme from Worthington Biochemical Corp. (COB-C 6IB) having a specific activity of 190 units/mg was used. This preparation contained 4 units/mg of carboxypeptidase A activity. A solution of carboxypeptidase B (1.9 units/ml) that had been exhaustively dialyzed against water was incubated with isocitrate lyase dialyzed as just described. Other details were closely parallel to those described for studies with carboxypeptidase A. The mass ratio of isocitrate lyase to carboxypeptidase B was a minimum of 34.

After centrifugation to remove the precipitate from trichloroacetic acid treatment, amino acid analyses were performed on the supernatants with a double-column Beckman–Spinco amino acid analyzer (120 C) equipped with range expander card and long-path cuvets. Detailed study of standard mixtures established that precision of ±3% could be expected at the 5-nmole level for all amino acids except arginine, serine, glycine, and alanine. The precision for these amino acids was poorer.

Studies of Bromomalonate Inhibition. Isocitrate lyase that had been activated and separated from GSH as described was diluted with eight volumes (0.8 ml) of 0.1 M Tris-HCl buffer containing 5 mM $MgCl_2$, pH 7.7 (25°). It was then incubated for 5 min at 30° either with 0.2 ml of 0.04 M DL-isocitrate prepared in 0.1 M Tris-HCl containing 5 mM $MgCl_2$, pH 7.7 (25°), or with 0.2 ml of the Tris-Mg^{2+} buffer alone. Then, to both series of tubes (*i.e.*, one preincubated with substrate and the other with buffer), 0.7 ml of 10.8 mM bromomalonate prepared in the Tris-Mg^{2+} buffer was added. After specific time periods at 30°, the enzymic reaction was initiated either with addition of 0.2 ml of 10 mM EDTA prepared in the Tris-Mg^{2+} buffer or by addition of this EDTA solution containing 0.04 M DL-isocitrate. The remainder of the assay was conducted in the usual way. For one control, an isocitrate lyase preparation was carried through the procedure described except that the Tris-Mg^{2+} buffer was substituted for bromomalonate during a 30-min preincubation period prior to initiation of the reaction.

For investigations of the pH dependence of bromomalonate inhibition, isocitrate lyase was activated and transferred into H_2O containing 1 mM $MgCl_2$ (Shiio *et al.*, 1965b). To 0.1-ml portions, 0.5 ml of 0.01 M potassium phosphate buffer of the specified pH was added followed by 0.2 ml of 18.9 mM freshly prepared bromomalonate (pH 7). After incubation at 30° for 30 min the enzymic reaction was initiated by addition of 1.2 ml of a mixture at 30° (pH 7.7) containing 90 μmoles of Tris,

[1] Abbreviation used that is not listed in *Biochemistry* 5, 1445 (1966), is: dansyl, 1-dimethylaminonaphthalene-5-sulfonyl.

18 μmoles of DL-isocitrate, 45 μmoles of $MgCl_2$, and 2 μmoles of EDTA. The usual enzyme assay was then completed. In controls, H_2O replaced bromomalonate during the preincubation with buffer of pH 7.85.

Effects of Guanidine Hydrochloride and Urea upon the Activity and Ultraviolet Spectrum of Isocitrate Lyase. First it was established that guanidine hydrochloride and urea at concentrations used in later experiments did not interfere with the colorimetric determination of glyoxylate (McFadden and Howes, 1960). In examinations of the effect of guanidine hydrochloride or urea upon isocitrate lyase, 0.2 ml of a solution containing 0.01 M Tris, 1 mM EDTA, 1 mM β-mercaptoethanol (pH 7.7, 25°), enzyme, and denaturing agent was incubated for 10 min at 25°. This solution was then treated for 10 min with 1.6 ml of a solution (pH 7.7) containing 200 μmoles of Tris, 6 μmoles of $MgCl_2$, and 20 μmoles of GSH to activate the enzyme. Then the standard enzymatic assay was performed. To examine the time course of action of these reagents, the procedure was conducted as just described except that incubation prior to activation was conducted for various times.

In one experiment, enzyme known to have an absorbancy ratio at 280 and 260 mμ of 1.70 was incubated at 28° in the presence of 0.8 M guanidine hydrochloride, 26 mM Tris, 0.6 mM EDTA, and 0.6 mM β-mercaptoethanol (pH 7.7, 25°). A spectrum in the ultraviolet range (light path 1.0 cm) was then periodically obtained in a Cary 14 recording spectrophotometer using a solution lacking enzyme as a blank. After 15 hr, the enzyme sample was dialyzed at 2° against two portions of 300 volumes of 0.05 M Tris (pH 7.7, 25°). Another sample that had not been treated with guanidine but had been treated in an otherwise identical manner served as a control. After dialysis the spectrum was again obtained for both samples and an aliquot of each was assayed for enzyme activity in the standard manner. A closely parallel experiment was conducted with 3 M urea.

Ultracentrifugal Analysis. Guanidine hydrochloride (obtained from Eastman Organic Chemicals, N. Y.) was recrystallized first from a hot absolute ethanol–benzene mixture and again from hot methanol by the method of Y. Nozaki (personal communication).[2] Fresh solutions were used. One molar solutions have a pH of 5. Because this is close to the isoelectric point of isocitrate lyase calculated from animo acid composition (Shiio *et al.*, 1965a), effects of protein charge upon equilibrium distribution by sedimentation (Tanford, 1961) were ignored. Immediately prior to all ultracentrifugal studies, enzyme solutions were equilibrated with the desired solution by dialysis against several changes (at least 300 volumes/change) of that solution at 2° over a 24–48-hr period. The final dialysate was used as a blank. Studies to examine carboxypeptidase A altered isocitrate lyase were conveniently conducted at 59,780 rpm at low protein concentrations (200 μg/ml) by employing ultraviolet optics in a Beckman–Spinco Model E analytical ultracentrifuge. Each print in a succession of photographs taken throughout sedimentation was then scanned in a Joyce densitometer to locate the sedimenting boundary. An approximate sedimentation coefficient was calculated.

Sedimentation equilibrium studies were conducted using the meniscus-depletion method of Yphantis (1964) and concentrations of isocitrate lyase of 0.015 and 0.02%. For these studies Rayleigh interference optics and sapphire cell windows were used with an ultracentrifuge equipped with electronic speed control. The special three-channel centerpiece (Beckman–Spinco) described by Yphantis permitted simultaneous studies of three solution–solvent pairs. Cells were loaded with sample and base fluid using a 100-μl Hamilton syringe with a lateral locking device to avoid mismatch of solution and solvent columns. Photographs taken during acceleration as suggested by Yphantis (1964) invariably revealed anomalous fringe displacements. At speed, the fringes were initially flat. After speed had been reached, photographs were taken using Spectroscopic II-G plates at suitable intervals to ascertain whether equilibrium had been reached. In all cases, speeds were selected that resulted in equilibrium distribution within 26 hr. Fringe displacement was measured by standard procedures using a Gaertner comparator and data were not considered significant unless there was no displacement in at least the upper half of the solution column.

In studies of subunits, densities of guanidine hydrochloride solutions at 20° reported by Kawahara and Tanford (1966) were used for the calculation of molecular weights. In studies of native enzyme, the density of the solvent was determined pycnometrically at 16° and corrected to 4° by assuming the same temperature dependence as that for pure water. A partial specific volume of 0.730 for isocitrate lyase (Shiio *et al.*, 1965a) was used for all calculations with a correction for temperature dependence (−0.001/2°) as necessary (Svedberg and Pedersen, 1940).

Electron Microscopy. Stock enzyme solutions of 0.3% were dialyzed against 0.01 M ammonium acetate. The resultant solution, freed of nonvolatile salts and mixed with four times its volume of 4% phosphotungstic acid (neutralized to pH 6.7 with NaOH), was sprayed on carbon-filmed grids. The grids were examined as soon as possible in either a Philips EM 100 or EM 200 electron microscope at an accelerating voltage of 80 kV. Point filaments were used.

Results

Specification of Concentration of Isocitrate Lyase. The relationship between absorbance (A) at 500 mμ after treatment with fresh Folin-Ciocalteu reagents and concentration of isocitrate lyase (using water as a blank) was: $(A - 0.025)/1.83$ = mg of isocitrate lyase/ml analyzed. This relationship, which was obtained in the protein concentration range of 35–200 μg/ml, was very similar to that for bovine serum albumin $(A - 0.025)/1.70$ obtained under similar conditions.

The Amino Terminus. Studies of treatment of isocitrate lyase and urea-treated isocitrate lyase with leucine aminopeptidase by procedures analogous to those de-

[2] Y. Nozaki, Department of Biochemistry, Duke University Medical Center, Durham, N. C.

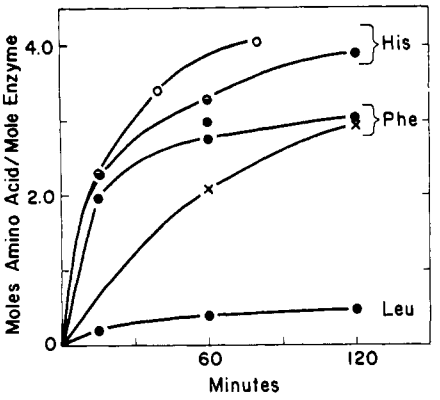

FIGURE 1: Amino acid release from isocitrate lyase (mol wt 206,000) as a consequence of digestion by carboxypeptidase A. The data are corrected for zero-time analyses of the isocitrate lyase–carboxypeptidase A mixture, although zero-time blanks for the three amino acids were almost negligible. A given symbol reflects the analysis of a given incubation mixture. In some cases analyses were performed in an experiment only with the short or long column thus accounting for incomplete analyses.

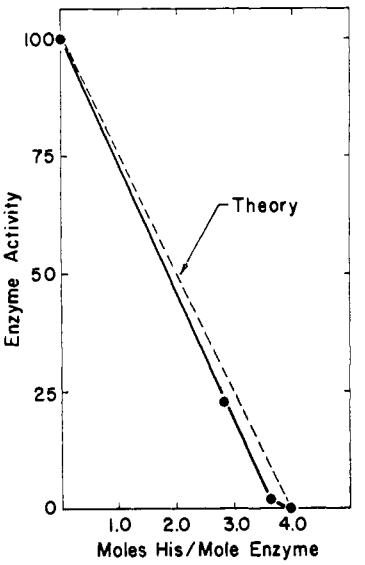

FIGURE 2: Relationship between histidine release by carboxypeptidase A digestion and isocitrate lyase activity. Data were obtained in an experiment depicted in Figure 1 (●—●) and are corrected for partial quenching (*ca.* 25%) by β-phenyl propionate which was not removed prior to assay of isocitrate lyase. The theoretical curve assumes a four-subunit model (mol wt 206,000) and assumes each subunit behaves as an independent catalytic entity.

scribed for carboxypeptidase A were uniformly unsuccessful in identifying the N terminus. Although serine (or asparagine), glycine, and aspartic acid increased slightly with time, in no case did the liberation of any of these amino acids exceed 0.8 mole/mole of isocitrate lyase in 90-min digestions and this yield did not further increase in the next 90 min. However, the zero-time controls consistently revealed the presence of *ca.* 1 mole of serine (or asparagine), 0.5 mole of glycine, and 0.5 mole of alanine per mole of isocitrate lyase. These amino acids were presumably present in a bound state such that they were not released by exhaustive dialysis or urea treatment but were freed by precipitation of isocitrate lyase with trichloroacetic acid. That methionine is the NH_2-terminal residue of isocitrate lyase was confirmed by several studies with dansyl chloride. In all experiments with dansyl chloride, dansyl chloride treated isocitrate lyase was precipitated with trichloroacetic acid prior to hydrolysis. In these experiments, the dansyl derivatives of glycine, serine, and alanine were undetectable but the methionine derivative was readily detected. Short hydrolysis of dansyl isocitrate lyase or its digestion with chymotrypsin (Gray, 1967) suggested that proline and tryptophan were not N terminal. Use of solvent C (Morse and Horecker, 1966) did not separate all dansyl-amino acids from dansylmethionine. Solvent D, however, unambiguously separated dansylmethionine from those derivatives not separated by solvent C. Use of both solvents suggested methionine as the N-terminal residue of isocitrate lyase. Since these solvents left mono-*O*-tyrosyl and ε-lysyldansyl derivatives as well as 1-dimethylaminonapthalene-5-sulfonic acid at the origin and the sulfonamide moved with the solvent front, there was little interference from these compounds. Solvent B (Nedkov and Genov, 1966) moved and separated all of the above compounds. Moreover, two distinct spots were observed with the dansylmethionine standard and corresponded in R_F with those for isocitrate lyase derived material. Presumably the minor spot was due to dansylmethionine sulfone.

The C Terminus. Hydrazinolysis revealed the release of histidine in a yield of 48%. The only other amino acids found (at much lower yields) with the Technicon automatic amino acid analyzer were serine, glycine, and alanine. These results, as well as those with dansyl chloride, leucine aminopeptidase, and carboxypeptidase A (next section), support the inference that these three amino acids were present in a bound state and were released with trichloroacetic acid.

Data from several digestions of different isocitrate lyase preparations by carboxypeptidase A are presented in Figure 1. The main variable in these experiments was the ratio of isocitrate lyase to proteolytic enzyme. It is clear from these and other experiments that histidine is released most rapidly and that the yield approaches a limiting value of 4 moles/mole of isocitrate lyase. The data suggest that histidine is at the carboxy terminus and support the same conclusion reached through hydrazinolysis studies. Phenylalanine is presumably penultimate. Phenylalanine may be followed by leucine although the data do not exclude the presence of two different types of chains. Zero-time controls in the experiments summarized in Figure 1, as well as analysis of the supernatant from trichloroacetic acid treated isocitrate lyase, consistently revealed the release of glycine, alanine, and serine. Isocitrate lyase was refractory to digestion by carboxypeptidase B.

Activity Loss with Removal of C Terminus. In a number of experiments, rapid activity loss has always been associated with the action of carboxypeptidase A upon isocitrate lyase. Data illustrating the quantitative relationship between histidine removal and activity loss

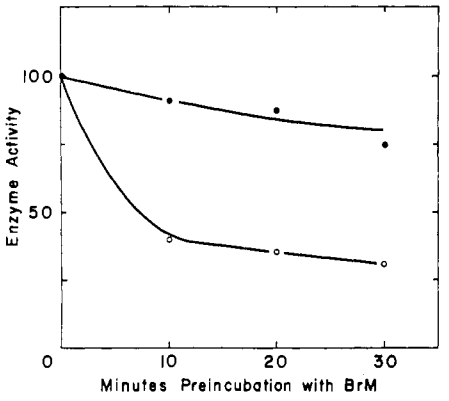

FIGURE 3: Substrate protection against 2-bromomalonate (BrM) inhibition. After preincubation for 5 min with the substrate DL-isocitrate, 2-bromomalonate was added and further incubation was conducted for the specified time before initiation of the enzymic reaction by addition of EDTA (●—●). In the other experiment, preincubation was conducted with buffer instead of substrate and substrate was added with EDTA to initiate the reaction after the specified incubation with 2-bromomalonate (○—○). See Materials and Methods for details.

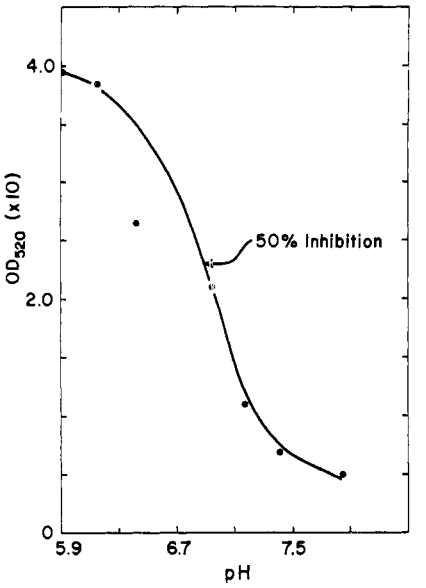

FIGURE 5: Activity recovered after 30-min preincubation with 2-bromomalonate as a function of pH. See Materials and Methods for details.

are represented in Figure 2. Data from controls incubated at 30° in the absence of carboxypeptidase A and transferred to β-phenyl propionate at appropriate intervals revealed no activity loss in the course of the experiment described.

In another experiment isocitrate lyase was treated for 90 min with carboxypeptidase A under conditions identical with those for one experiment shown in Figure 2 (●——●). Carboxypeptidase A was then separated from isocitrate lyase by gel filtration. The altered isocitrate lyase had a residual activity of 4% of that of native enzyme after this treatment. A portion of the altered enzyme was then submitted to sedimentation analysis as described. Estimation of a rough sedimentation coefficient, $s_{20,w}$, yielded a value of 10 S for altered enzyme, which is the same within experimental error as that for native isocitrate lyase (Shiio *et al.*, 1965a).

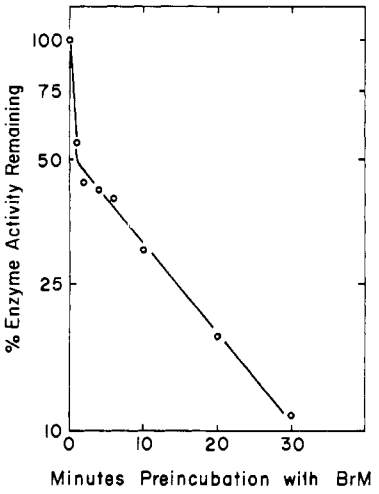

FIGURE 4: Semilog plot of per cent remaining enzyme activity *vs.* time of exposure at 30° of isocitrate lyase to 4.4 mM BrM in the absence of substrate in the standard incubation mixture supplemented with 1 mM EDTA. Reactions were initiated by the addition of substrate and the standard assay was performed.

Bromomalonate Inhibition. Figure 3 illustrates the time course of inhibition by 4.2 mM 2-bromomalonate, an analog of a competitive inhibitor of isocitrate cleavage, malonate (Rao and McFadden, 1965), and the protection afforded by 2.2 mM substrate. In other experiments, 2-bromomalonate was incubated with isocitrate lyase for 30 min under the standard conditions and was then separated from the enzyme by Sephadex gel filtration. When assayed in the presence of EDTA this preparation had 25% of the activity of a control that had not been treated with inhibitor but had been manipulated in an otherwise identical manner. This established that the inhibition was irreversible. Results depicted in Figure 4 suggest that the inhibition is biphasic.

Figure 5 displays the pH dependence of inhibition by 4.2 mM 2-bromomalonate. From the pK_a's of malonic acid and the known acid-strengthening effect of an α-bromo substituent (Albert and Serjeant, 1962), it is clear that 2-bromomalonate dianion predominated throughout the pH range. At pH 6.8, 50% inhibition occurs.

Dissociation of Isocitrate Lyase into Subunits. Results of studies of the inhibition of isocitrate lyase by various concentrations of guanidine hydrochloride and urea are summarized in Table I. Because the guanidine hydrochloride and urea were diluted *ca.* 1:10 after the preincubation during enzyme activation and assay (20 min), the observed inhibition by low concentrations of guanidine hydrochloride was especially striking. It suggested that the effects of guanidine hydrochloride were not markedly reversed by dilution and subsequent short incubation at 30°. Separate studies with 1 M NaCl in which essentially no inhibition was observed established that the effects of guanidine hydrochloride were not simply

TABLE I: Effect of Guanidine Hydrochloride and Urea upon the Activity of Isocitrate Lyase.

Concn (M) of the Inhibitor during Preincubn[a]	% Inhibition with	
	Guanidine Hydrochloride	Urea
0.1	34	0
0.2	57	0
0.4	72	0
0.8	88	5
1.6	100	10
2.0	100	27
2.4	100	37
2.6	100	40
2.8	100	83
3.0	100	96
3.2	100	96
4.0	100	97

[a] Preincubation period was 10 min.

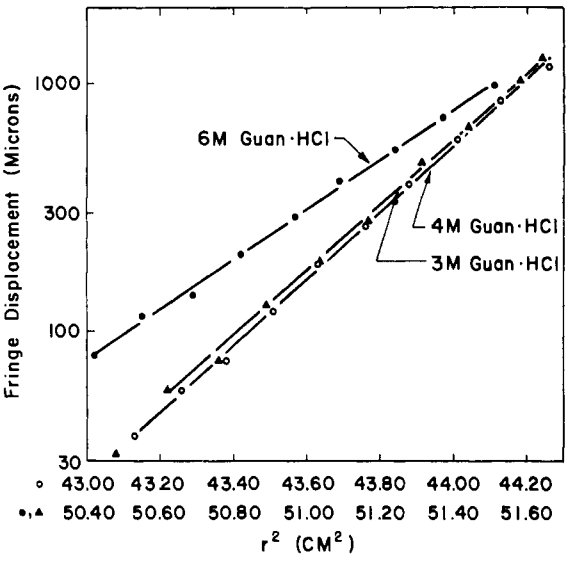

FIGURE 6: Semilog plots reflecting the equilibrium distribution at 20° of 0.03% isocitrate lyase in 0.1 M β-mercaptoethanol plus the indicated additions. The speeds for experiments with 3, 4, and 6 M guanidine hydrochloride were 36,112, 36,122, and 36,122 rpm, respectively.

nonspecific salt effects exerted by ionic strength elevation. In studies similar in design to those described in Table I, the mode of inhibition by guanidine hydrochloride was examined at molar concentrations of 0.1 and 0.2, respectively. This reagent was found through Lineweaver–Burk (1934) analysis to be a noncompetitive inhibitor with a K_I of 0.20 M. Since high concentrations of urea were required for inhibition, the mode of action of urea was not thoroughly examined.

Next, the time course of action of these reagents was investigated. The inhibition with 0.2 and 0.8 M guanidine hydrochloride appeared to be virtually instantaneous being maximal after 15-sec preincubation with isocitrate lyase. In contrast, the onset of inhibition by 2.4 M urea was much slower increasing from 10% after 2-min preincubation to 51% after 10-min preincubation. Although the data are not shown, separate studies in which 0.2 M guanidine hydrochloride was also used in the preincubation phase but in which the activation phase was eliminated, *i.e.*, EDTA was substituted for GSH (Shiio *et al.*, 1965b), also resulted in very rapid inhibition. However the degree of inhibition was *ca.* 30% less suggesting slight time-dependent reversal of inhibition by dilution.

Although treatment of isocitrate lyase with 0.8 M guanidine hydrochloride results in 88% inhibition, there is no attendant spectral alteration between 320 and 260 mμ nor is there a change in sedimentation coefficient. The original activity (74% of it) could be recovered by dialysis to remove guanidine hydrochloride. Clearly inhibition at lower concentrations of guanidine involves subtle structural alteration of isocitrate lyase.

Treatment of isocitrate lyase with 3 M urea, under conditions that completely inactivate isocitrate lyase (Table I), also results in no spectral change. However, the observed inactivation cannot be reversed by urea removal through dialysis. In other experiments it has been established that the enzyme slowly precipitates in 8 M urea and cannot be redissolved by removal of urea.

Studies with guanidine hydrochloride were expanded to include concentrations in the range of 2–6 M. Dissociation was incomplete at 2 M guanidine hydrochloride plus 0.1 M β-mercaptoethanol but was obtained in the

TABLE II: Molecular Weight of Native Isocitrate Lyase and the Subunit by the Yphantis Meniscus-Depletion Technique.

Sample	App Mol Wt[b] (g)
Native isocitrate lyase (IL)[a]	
0.015%	204,700
0.02%	207,500
IL + 3 M guanidine hydrochloride + 0.1 M β-mercaptoethanol	47,800
IL + 4 M guanidine hydrochloride + 0.1 M β-mercaptoethanol	49,800
IL + 6 M guanidine hydrochloride + 0.1 M β-mercaptoethanol	47,670

[a] These studies were conducted on the indicated concentrations of isocitrate lyase that had been equilibrated through dialysis at 2° with 0.10 M Tris-HCl, pH 7.7 (20°), containing 5 mM $MgCl_2$. The dialysate served as a blank. [b] Estimated with the aid of analysis of semilog plots of the type shown in Figure 6 by the method of least squares using a computer program written by Mr. K. J. Johnson, Washington State University, and the IBM 360(67) computer. The standard deviations of each value from top to bottom were 8.7, 6.6, 7.3, 10.0, and 7.8%, respectively.

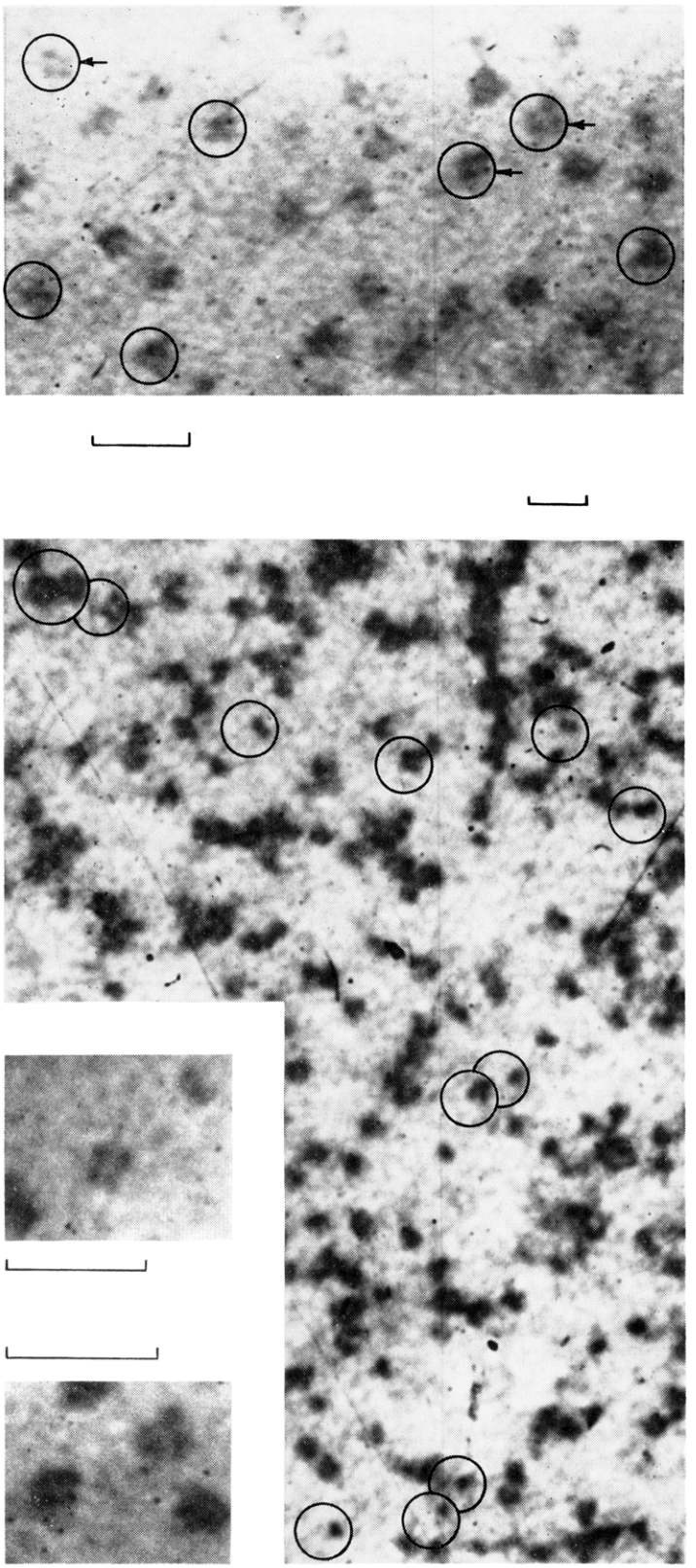

FIGURE 7: Studies of negatively stained isocitrate lyase using the Philips EM 200 electron microscope. In all cases the distance between calibration marks is 200 Å. The top figure represents the examination of a fresh enzyme preparation. Structures with arrows were further magnified and are shown in the insets. The bottom figure represents examination of an isocitrate lyase preparation that had been stored at 2° in 0.01 M ammonium acetate for 3 days before examination. This mode of storage leads to some dissociation as shown by the presence of dimers and monomers. The figures were reproduced from reversed-contrast prints.

range of 3–6 M guanidine hydrochloride plus 0.1 M β-mercaptoethanol. Dissociation into components of uniform size is attested to by the linearity (Figure 6) of the plots of ln (fringe displacement) *vs.* (radius of centrifugation)^2 (Yphantis, 1964).

The data from the meniscus-depletion studies partially described in Figure 6 were used to calculate the apparent molecular weight of the subunit. These results and others relating to native isocitrate lyase are tabulated in Table II. In none of these studies was there any indication of heterogeneity.

Figure 7 shows the results of electron microscopic examination of isocitrate lyase.

Discussion

The data from sedimentation equilibrium suggest that the molecular weight of isocitrate lyase is about 206,000. This value is somewhat high because it is uncorrected for the negative charge borne by isocitrate lyase at pH 7.7. The magnitude of this charge can be estimated from the amino acid composition (Shiio *et al.*, 1965a) and is *ca.* 50. The downward correction is small and depends in part upon the partial specific volume of components furnishing counterion (Tanford, 1961). If it is assumed that the main counterion was Tris cation, the maximum correction downward is 4000. This is based upon a value of 0.80 for the partial specific volume of Tris-HCl at 0.1 M (unpublished data). The corrected molecular weight of isocitrate lyase becomes 202,000. This is lower than the uncorrected value of 222,000 reported earlier. That value which was based upon measurements of sedimentation velocity and diffusion was less reliable. It is of some interest that the molecular weight of isocitrate lyase from *Chlorella pyrenoidosa* is 170,000 as measured by sedimentation velocity and diffusion (John and Syrett, 1967).

At first sight, the data support dissociation of isocitrate lyase into four subunits in the presence of 3–6 M guanidine hydrochloride plus 0.1 M β-mercaptoethanol. However, the molecular weights reported here are uncorrected for preferential interaction of the unfolded protein with guanidine hydrochloride or water (Schachman and Edelstein, 1966). The development of reliable techniques to estimate preferential interaction is still in its infancy. The four-subunit model receives support from the carboxypeptidase A digestions in which the yield of carboxy-terminal histidine seems to approach a limiting value of 4 moles/mole of isocitrate lyase.

Additional support for a four-subunit model of isocitrate lyase is derived from studies with the electron microscope. Frequently seen structures are tetrameric and approximately square or rhomboidal in appearance. In the best defined structures, four roughly spherical or elliptical subunits are apparent. The apparent presence of dimers in one figure is also compatible with tetrameric units that have dissociated into halves. The apparent presence of monomers in the same figure is consistent with dissociation. The triangular forms that are occasionally seen in addition to the four-membered configurations mentioned are compatible with a tetrahedral array of subunits. The average radius of roughly spherical subunits in the best figures obtained with the electron microscope is about 25 Å. This corresponds to a molecular weight of about 55,000/subunit. Considering the uncertainty of this estimate, it is in reasonable accord with other experimental results.

Data presented permit the inference that the subunits are similar. For example, there is no detectable difference in molecular weight. Subunits are terminated at the carboxyl end by histidine and methionine may be the sole N-terminal residue. Regrettably, however, the information presented does not establish whether or not the subunits are identical. Fingerprinting of tryptic peptides as well as those obtained by cyanogen bromide is underway as are efforts to separate the subunits.

Of considerable interest is the observation that loss of catalytic activity by isocitrate lyase closely parallels the removal of C-terminal histidine by carboxypeptidase A. This suggests that each subunit behaves like an independent catalytic entity. Indeed there is no kinetic evidence to date that is compatible with cooperativity between subunits of the enzyme from *P. indigofera* (McFadden and Howes, 1963; Rao and McFadden, 1965). The data relating loss of catalytic activity to removal of carboxy-terminal residues are reminiscent of similar phenomena occurring with class I aldolases. Daron *et al.* (1966) have tentatively classified isocitrate lyase as a class II aldolase on the basis of the thiol and metal ion requirements. Whether isocitrate lyase bears any structural relationship to aldolases is of deep significance and must await further experimentation.

Carboxy-terminal histidine may be directly involved in catalysis or simply required for maintenance of the correct configuration of catalytic sites. Although a choice cannot be made between these alternatives, the data do rule out any change in quaternary structure and major changes in secondary or tertiary structure as a consequence of histidine removal. In contemplating the mechanism of the isocitrate lyase catalyzed reaction, it seems likely that participation of a basic enzyme residue is required. The observed catalysis of glyoxylate-promoted exchange of succinate hydrogens with the medium (Daron *et al.*, 1966) may well involve a basic functional group.

The pH dependence of inhibition by 2-bromomalonate, an anolog of the competitive inhibitor malonate, is compatible with alkylation of histidine(s). The kinetics of inactivation are consistent with alkylation of two different types of residues, or a single type of residue in two environments. It will be interesting to determine whether histidine is in fact the sole type of residue modified and, if it is, to determine whether the histidine is carboxy terminal. Whatever the outcome, the observed protection against bromomalonate inhibition by substrate may open the way to selective labeling of the catalytic site(s).

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