Isocitrate Lyase from *Cephalosporium acremonium*. Role of Mg^{2+} Ions, Kinetics, and Evidence for a Histidine Residue in the Active Site of the Enzyme†

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ABSTRACT: Isocitrate lyase was purified from *Cephalosporium acremonium* CW-19 from cultures growing with poly(oxyethylene)sorbitan monopalmitate as the carbon source. Its subunit M_r and native M_r were 63 000 ± 2000 and 250 000 ± 5000, respectively. We found the Mg^{2+}−isocitrate complex to be the true substrate and that Mg^{2+} ions act as a nonessential activator, according to the model reported by Giachetti et al. (1988) [Giachetti, E., Pinzauti, G., Bonaccorsi, R., & Vanni, P. (1988) *Eur. J. Biochem. 172*, 85−91], from which the kinetic parameters were calculated. The kinetic study is consistent with an ordered Uni-Bi mechanism, and the kinetic and rate constants of the model were calculated. pH dependence of the cleavage reaction indicated that the catalysis was dependent on two dissociable groups on the enzyme−substrate complex. The enzyme was inactivated by diethyl pyrocarbonate following first-order kinetics at all reagent concentrations used. The pseudo-first-order rate constant of inactivation increases with pH, suggesting participation of an amino acid residue with p*K* 6.0. Hydroxylamine added to the inactivated enzyme quickly restored the incremental absorption at 240 nm and most of the activity. Data analyses indicated that diethyl pyrocarbonate inactivation is a consequence of modification of 11 histidine residues per enzyme subunit, and from statistical analysis, we concluded that one is catalytically important. Mg^{2+}−isocitrate protects the enzyme against diethyl pyrocarbonate inactivation with a K_s value of 26.8 ± 2.1 μM, close to the K_m value. Isocitrate protects the enzyme but a high concentration, suggesting its binding to the catalytic site of the nonactivated enzyme. Mg^{2+} ions also produced total competitive protection.

Isocitrate lyase (EC 4.1.3.1) is a key enzyme of the glyoxylate pathway, catalyzing the reversible aldol cleavage of *threo*-D_s-isocitrate to succinate and glyoxylate. This pathway has been formed in many forms of life (Cioni et al., 1981) and enables microorganisms to grow on C_2 compounds by generating the precursors necessary for biosynthesis. Isocitrate lyase has been characterized from several sources as a tetrameric enzyme requiring Mg^{2+} ions for catalysis [see for a review Vanni et al. (1990)], but its role in kinetics has been reported in only a few cases. For the isocitrate lyase from *Pinus pinea*, Giachetti et al. (1988) and Giachetti and Vanni (1991) have demonstrated that the Mg^{2+}−isocitrate complex is the true substrate and that Mg^{2+} acts as a nonessential activator. We have also reported a similar behavior for the *Phycomyces blakesleeanus* isocitrate lyase (Rúa et al., 1990). On the other hand, the identity of amino acid residues involved the active site of isocitrate lyases has been the subject of many studies (Ko & McFadden, 1990a; Ko et al., 1991; Rúa et al., 1992: Olano et al., 1992; Diehl & McFadden, 1994).

C. acremonium is a filamentous fungus, economically and medically important since it produces the β-lactam antibiotic cephalosporin C. Its biosynthesis from L-α-aminoadipic acid, L-cysteine, and L-valine has been well characterized (Abraham, 1986; Queener & Neuss, 1982). The knowledge of the regulation of the biosynthetic pathway, especially with regard to the control of α-aminoadipic acid, which starts the formation of both penicillins and cephalosporin C, is still scarce (Hoskins et al., 1990). Since this α-aminoadipic acid is synthesized in *C. acremonium* from both 2-oxoglutarate and acetyl-CoA, we decided to investigate the regulation of isocitrate flux in this fungus. To date, little has been learned about the regulation of isocitrate flux in filamentous fungi (Hilgenberg et al., 1987; Rúa et al., 1989, 1990). We have purified NADP−isocitrate dehydrogenase in *C. acremonium* and studied its regulatory properties (data to be published elsewhere). In this paper, we report the purification and characterization of isocitrate lyase from *C. acremonium*. In addition, from kinetic studies we were able to determine the true substrate of the enzyme, the role of Mg^{2+} ions in enzyme kinetics, and the mechanism. From pH dependence studies and from diethyl pyrocarbonate modification of the enzyme, we were also able to deduce the role played by histidyl residue(s) on the catalytic mechanism of *C. acremonium* isocitrate lyase.

EXPERIMENTAL PROCEDURES

Materials. *threo*-DL-Isocitrate (trisodium salt), *threo*-D_s-isocitrate (monopotassium salt), succinic acid, glyoxylic acid, phenylhydrazine hydrochloride, sodium dodecyl sulfate (SDS),[1] DEAE-Sephacel, diethyl pyrocarbonate (DEP), and Blue Dextran were purchased from Sigma Chemical Co.,

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[1] Abbreviations: DEP, diethyl pyrocarbonate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, 1,4-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Mes, 4-morpholineethanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid; PAGE, polyacrylamide gel electroporesis; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate.

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St. Louis, MO. Sephadex G-200, M_r markers for analytical gel filtration, and M_r markers for SDS/PAGE were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Reagents for gel electrophoresis were from Shandon Southern Products, Cheshire, U.K. Pig heart isocitrate dehydrogenase (EC 1.1.1.42), $NADP^+$ (disodium salt), Mops, imidazole, Mes, dithiothreitol, and phenylmethanesulfonyl fluoride were from Boehringer, Mannheim, Germany. Tween 40 and all other chemicals used were standard analytical grade and provided by Merck, Darmstadt, Germany.

Cell Growth. We used the *C. acremonium* strain CW-19 (*Acremonium chrysogenum* ATCC 36225), grown essentially as described by Shen et al. (1986), except that we used Tween 40 as carbon source in the defined fermentation medium to induce isocitrate lyase activity (data not shown) and 250 mL Erlenmeyer flasks to develop inoculum and for fermentations. It was incubated on slant medium to induce sporulation for 9 days, a spore suspension being used to inoculate seed medium no. 1. The defined fermentation medium (40 mL) in 250 mL Erlenmeyer flasks was then inoculated with 2 mL aliquots of seed culture. Adjusted to pH 7.4, it contained in a final volume of 1000 mL of distilled water 20 mL of Tween 40 [poly(oxyethylene)sorbitan monopalmitate $d^{20} = 1.085$] as carbon source and 3.2 g of DL-methionine, 12 g of L-asparagine, 8 mL of 2% (NH_4)-$Fe(SO_4)_2$, and 144 mL of a stock salt solution. This stock solution contained the following in a volume of 1000 mL: 104 g of KH_2PO_4, 102 g of KH_2PO_4, 5.87 g of Na_2SO_4, 2.4 g of $MgSO_4 \cdot 7H_2O$, 0.2 g of $ZnSO_4 \cdot 7H_2O$, 0.2 g of $MnSO_4 \cdot H_2O$, 0.05 g of $CuSO_4 \cdot 5H_2O$, and 0.3775 g of $CaCl_2$. All incubations were then made in a Psychrotherm G-27 orbital shaker (New Brunswick Scientific Co., Inc.) at 250 rpm and 25 °C. After 2 days, coincident with the highest specific isocitrate lyase activity, the culture was filtered, and the mycelia were harvested. For enzyme purification, mycelia were frozen on liquid nitrogen.

Isocitrate Lyase Purification. The source for enzyme purification was 48 h old mycelia of *C. acremonium* grown as described above. The enzyme was purified following the procedure described by Rúa et al. (1990), but with the insertion of a hydrophobic chromatography step and a FPLC ionic exchange step. All the purification steps were carried out at 4 °C. The mycelium mass collected by filtration was cut into pieces and suspended in 50 mM imidazole hydrochloride buffer, pH 7.5, containing 10 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM $MgSO_4$, and 1 mM PMSF (buffer A) at a ratio of 5 mL/g wet weight, and homogenized with glass beads in a Braun MSK homogenizer cell disrupter for two 30 s periods with a 15 s interval. Glass beads and unbroken cells were removed by centrifugation at 1500*g* for 5 min. The homogenate was centrifuged at 104000*g* for 45 min. The supernatant was adjusted to 30% saturation with solid $(NH_4)_2SO_4$ added slowly. After stirring for 20 min and centrifuging at 15000*g* for 20 min, we added further $(NH_4)_2$-SO_4 to the supernatant to give 60% saturation. The preparation was stirred and centrifuged as above, and the pellet, which contained the isocitrate lyase activity, was suspended in a small volume of 20 mM imidazole hydrochloride buffer pH 7.0, containing 5 mM KCl, 1 mM EDTA, 1 mM $MgSO_4$, and 1 mM dithiothreitol (buffer B). The suspension was loaded (~3 mL) onto a Sephadex G-200 column (3.5 cm × 50 cm) equilibrated with buffer B. Isocitrate lyase fractions (2.5 mL) with at least half of the maximum activity were pooled, and all this material was chromatographed on a DEAE-Sephacel column (2 cm × 23 cm) equilibrated with buffer B. The column was washed with the same buffer B until the absorbance at 280 nm had returned to base line, and the isocitrate lyase activity was eluted with a linear gradient of 0−0.25 M KCl in buffer B (total volume 150 mL). Powdered $(NH_4)_2SO_4$ was added to the pooled fractions containing half of the maximum isocitrate lyase activity, to give a concentration of 1 M. The solution (15 mL) was loaded onto a phenyl-Sepharose column (1.5 cm × 15 cm) equilibrated with buffer B containing 0.9 M $(NH_4)_2SO_4$. The column was washed with the same buffer until the absorbance at 280 nm had returned to base line, and the enzymatic activity was eluted with a linear gradient of 0.9−0 M $(NH_4)_2$-SO_4 in buffer B (total volume 100 mL). Active fractions were pooled, and afterward desalted and concentrated with a Centricon-30 microconcentrator from Amicon. The desalted sample in buffer B was applied in 5 mL fractions to a DEAE-5PW (8.0 mm × 75 mm) column for FPLC (Waters 600E apparatus). The column was equilibrated and washed with the same buffer until the absorbance at 280 nm had returned to base line, and the isocitrate lyase activity was eluted at a flow rate of 1 mL/min with a linear gradient of 0−0.25 M KCl in buffer B (total volume 50 mL). Active fractions (~10 mL) containing isocitrate lyase activity were pooled and dialyzed for 24 h against 1000 mL of 50 mM Mops/NaOH, pH 7.3, containing 1 mM EDTA, 1 mM benzamidine hydrochloride, 1 mM dithiothreitol, 1.2 mM PMSF, and 40% glycerol.

Analytical Gel Filtration. An FPLC column (8.0 mm × 300 mm) of Silicagel-300SW was equilibrated with 0.1 M sodium phosphate buffer, pH 6.7, containing 0.1 M Na_2SO_4 and 0.05% NaN_3, at a flow rate of 0.8 mL/min. The markers used were ferritin (M_r 450 000), catalase (M_r 232 000), aldolase (M_r 158 000), BSA (M_r 68 000), ovalbumin (M_r 45 000), and chymotrypsinogen A (M_r 25 000).

PAGE. PAGE under nondenaturing conditions was performed on gel slabs [7.5% acrylamide and 0.15% bis-(acrylamide) at pH 9.5] by a modification of the methods of Bryan and Davis as described in Sigma Technical Bulletin No. MKR-137 (1986), with a Bio-Rad Mini-protean II apparatus modified by using special spacers which allowed us to obtain a gel with a thickness of 1.5 mm. SDS/PAGE was carried out on gel slabs (Mini-protean II) with 12% acrylamide and 0.1% of SDS in Tris/HCl/SDS buffer according to the Laemmli method (Laemmli, 1970). The subunit M_r markers used were trypsin inhibitor (M_r 20 100), carbonic anhydrase (M_r 30 000), ovalbumin (M_r 43 000), BSA (M_r 67 000), and phosphorylase *b* (M_r 94 000).

Isocitrate Lyase Assays. Isocitrate lyase for the cleavage reaction was assayed at pH 6.8 and 30 °C by a modification of the continuous method described by Dixon and Kornberg (1959) as indicated by Rua et al. (1990). Formation of the phenylhydrazine derivative of glyoxylate was monitored at 324 mm ($\epsilon = 14.63 \times 10^3\ M^{-1}\ cm^{-1}$). A unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of glyoxylate phenylhydrazone/min under standard assay conditions. The condensation reaction catalyzed by isocitrate lyase was assayed by coupling the formation of isocitrate to the reduction of $NADP^+$ by using isocitrate dehydrogenase at pH 7.5 and 30 °C, as described by Rúa et al. (1990). The NADPH formation was monitored by the change in absorbance at 340 nm ($\epsilon = 3.26 \times 10^3$

M^{-1} cm^{-1}). A unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of NADPH/min under standard conditions. Specific activity refers to units of enzyme activity per milligram of protein.

Protein concentration was measured by the method of Bradford (1976) or by Warburg and Christian's method described by Layne (1957). BSA was used as standard.

Data Analysis. In all kinetic experiments, we used *threo*-D_s-isocitrate and the enzymatic preparation obtained as the eluate of phenyl-Sepharose chromatography. The concentration of Mg^{2+}–isocitrate complex was calculated by using a stability constant (K_o) for the association reaction of Mg^{2+}–isocitrate complex of 1350 M^{-1} at pH 7.4 (Duggleby & Dennis, 1970). The value of this constant was corrected for each pH according to the dissociation constants of isocitric acid, as described by Giachetti et al. (1988). Linear and nonlinear fitting of the kinetic data to the corresponding equation was carried out using either the Enzfitter (Leatherbarrow, 1987) or the Sigmaplot for Windows (Jandel Scientific, 1993) program. We have adopted Segel's kinetic nomenclature (Segel, 1975b).

We plotted and fitted initial velocity data obtained at each pH value by varying the concentrations of Mg^{2+}–isocitrate to the Michaelis–Menten equation after graphically checking that the double-reciprocal plots were linear. The variation of the values for V_{max} and V_{max}/K_m as a function of pH was fitted to the log form of the equations:

$$V_{max_{pH}} = \frac{V_{max}}{1 + [H^+]/K_A + K_B/[H^+]} \quad (1)$$

$$\frac{V_{max_{pH}}}{K_{m_{pH}}} = \frac{V_{max}/K_m}{1 + [H^+]/K_A + K_B/[H^+]} \quad (2)$$

where K_A and K_B are the dissociation constants for ionizable groups on the enzyme.

For the study of the Mg^{2+} activation system, the initial velocity data were fitted to the Michaelis–Menten equation according to the activation model described by Giachetti et al. (1988), by means of the analysis of the dependence of $V_{max_{app}}$ and $K_{m_{app}}$ parameters on free Mg^{2+} concentration using the equations (Giachetti & Vanni, 1991):

$$\frac{1}{V_{max_{app}} - V_{max}} = \frac{1}{V_{max}(\beta - 1)} + \left[\frac{\alpha K_a}{V_{max}(\beta - 1)}\right]\frac{1}{[Mg^{2+}]} \quad (3)$$

$$K_{m_{app}}(\alpha K_a + [Mg^{2+}]) = \alpha K_{sa} K_a + \alpha K_{sa}[Mg^{2+}] + \left(\frac{\alpha K_{sa}}{K'_{ea}}\right)[Mg^{2+}]^2 \quad (4)$$

where subscript "sa" refers to Mg^{2+}–isocitrate complex and "a" to Mg^{2+}; K_{sa}, the equilibrium constant for substrate (SA) dissociation from the E(SA) complex; K_a, the equilibrium constant for Mg^{2+} dissociation from the activated enzyme (AE); K'_{ea}, the equilibrium constant for Mg^{2+} dissociation from the catalytic site of the AEA complex; K'_{sa}, the equilibrium constant for substrate (SA) dissociation from the AE(SA) complex; α, the K'_{sa}/K_{sa} ratio; V_{max}, the maximal velocity of the nonactivated enzyme (E); V'_{max}, the maximal velocity of the activated enzyme (AE); and β, the V'_{max}/V_{max} ratio. For a more detailed description of the model and the equation, see Giachetti and Vanni (1991) and Segel (1975a).

Scheme 1

$$E + A \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \left[EA \rightleftharpoons EPQ\right] \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} P + EQ \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} E + Q$$

For the analysis of the kinetic mechanism followed by the isocitrate lyase, initial-rate data were plotted in double-reciprocal form and, after a check to ensure that these plots were linear, were fitted to the Michaelis–Menten equation. Slopes and intercepts in secondary replots were fitted to straight lines by linear regression. Initial-rate data were also fitted to eq 5 which described the complete velocity equation

$$v = V_{max_f}\{[A] - [P][Q]/K_{eq}\}/\{K_{m_A} + [A] + V_{max_f}K_{m_A}[P]/V_{max_r}K_{eq} + V_{max_f}K_{m_P}[Q]/V_{max_r}K_{eq} + [A][P]/K_{ip} + V_{max_f}[P][Q]/V_{max_r}K_{eq}\} \quad (5)$$

for an ordered Uni-Bi mechanism (Scheme 1) according to Segel (1975b), where A represents Mg^{2+}–isocitrate complex, P represents succinate, Q represents glyoxylate, and V_{max_f} and V_{max_r} are the maximum velocities for the cleavage reaction and condensation reaction, respectively.

Inactivation of Isocitrate Lyase with Diethyl Pyrocarbonate. A solution of diethyl pyrocarbonate was prepared before each experiment by diluting it in absolute ethanol. The concentration of diluted diethyl pyrocarbonate was determined spectrophotometrically by reaction with imidazole. An aliquot of the dilution was added to 7.5 mM imidazole at pH 7.3, and the concentration was calculated from the increase in absorbance in the range of 230–240 nm due to the formation of *N*-carbethoxyimidazole residues, using an extinction coefficient of 3200 M^{-1} cm^{-1} (Ovádi et al., 1967). Purified *C. acremonium* isocitrate lyase was devoid of glycerol after four passages in a Centricon-30 microconcentrator from Amicon with 50 mM Mops/NaOH, pH 7.3, containing 1 mM EDTA. The reaction of isocitrate lyase with diethyl pyrocarbonate was carried out at 20 °C by incubating isocitrate lyase (subunit concentration 1.2–1.5 μM) in Eppendorf tubes with DEP in 50 mM Mops/NaOH, pH 7.3, containing 1 mM EDTA. The concentration of ethanol present during the activity assay (0.8% v/v) had no effect upon enzyme activity and stability. Aliquots (10 μL) were withdrawn at intervals and assayed for enzyme activity. The first-order rate constant for the decomposition of DEP (k') was calculated by measuring the time-dependent decomposition of DEP in a separate control experiment run as described above. The value of k' for DEP decomposition was determined at each pH value. For experiments of the pH dependence of inactivation, we used 50 mM Mes/NaOH buffer in the pH range 5.5–6.75, and 50 mM Mops/NaOH buffer in the pH range 7.0–7.75, both containing 1 mM EDTA.

UV Difference Spectra and Stoichiometry of Histidine Modification. Spectroscopic studies were performed at 20 °C in 50 mM Mops/NaOH buffer, pH 7.3, containing 1 mM EDTA, starting with DEP at 0.25 mM and isocitrate lyase at 2.5 μM subunit concentration. The recorded absorbance was computer-corrected for the blank control (enzyme in buffer without DEP) in the range of 220–290 nm for each incubation period. The increase in absorbance at 240 nm

Table 1: Purification of Isocitrate Lyase from the Mycelium of *C. acremonium* CW-19[a]

fraction	protein (mg)	total activity (units)	specific activity (units/mg)	purification factor (*x*-fold)	yield (%)
homogenate	1381	88.3	0.06	1	100
104000*g* supernatant	751	68.4	0.09	1.5	75
$(NH_4)_2SO_4$ fractionation 30−60% saturation	521	56.2	0.11	1.8	64
Sephadex G-200 eluate	76	26.6	0.35	5.8	30
DEAE-Sephacel eluate	19	11.6	0.61	10.2	13
phenyl-Sepharose eluate	4	10.3	2.57	42.8	12
DEAE-5PW eluate	0.18	6.3	35	583.3	7.1

[a] The homogenate was obtained from 20 g of mycelia. For experimental details, see the text.

using an extinction coefficient of 3200 M^{-1} cm^{-1} (Ovádi et al., 1967) allowed us to determine the stoichiometry of the formation of the *N*-carbethoxyhistidine residues (Miles, 1977).

Hydroxylamine Treatment. After 30 min of incubation of the isocitrate lyase (2.5 μM subunit concentration) at 20 °C with 0.25 mM DEP in 50 mM Mops/NaOH buffer, pH 7.3, containing 1 mM EDTA, hydroxylamine was added to a final concentration of 0.5 M. We then determined the differential absorbance at 240 nm of the sample treated with DEP and the control (enzyme alone).

Restoration of Activity Using Hydroxylamine. After the inactivation of isocitrate lyase with varying concentrations of DEP and a reaction time of 10 min, excess DEP was destroyed by adding 5 mM histidine (pH 7.0). Then the samples were treated with 0.5 M hydroxylamine (pH 7.0) at 20 °C for 30 min. Following the completion of this time, the hydroxylamine excess of the samples was eliminated by ultrafiltration and buffer change using an Amicon Centricon-30 microconcentrator, and the samples were then assayed for isocitrate lyase activity using the condensation reaction assay (Rúa et al., 1990). Controls using unmodified enzyme treated with hydroxylamine were run simultaneously.

RESULTS AND DISCUSSION

Purification and Characterization of Isocitrate Lyase. The results of a typical purification experiment of isocitrate lyase from the mycelium of *C. acremonium* are summarized in Table 1. The more effective steps at the purification procedure were the last two, the hydrophobic and the ionic exchange chromatography. Isocitrate lyase activity eluted as a single peak from the phenyl-sepharose chromatography when the ammonium sulfate concentration was 0.15 M, which indicates a high degree of hydrophobicity for the enzyme, as for the enzyme from *Pinus pinea* (Pinzauti et al., 1986). The last step of FPLC ionic exchange assures a great increase in the specific activity and obviously in the purification factor. The yield and final purification factor were 7% and 583-fold, respectively. The specific activity of *C. acremonium* isocitrate lyase found by us is among the highest reported and similar to those described for the enzyme from *E. coli* ML 308 (MacKintosh & Nimmo, 1988), from *Chlorella pyrenoidosa* (John & Syrett, 1967), and from *Pseudomonas indigofera* (Shiio et al., 1965).

The purified *C. acremonium* isocitrate lyase seems to be homogeneous judging from the existence of a single protein band with a relative mobility of 0.22 observed on PAGE (7.5% acrylamide) under nondenaturing conditions (data not shown). A high degree of homogeneity was also indicated by using PAGE in the presence of SDS, as evidenced by the existence of a single band with a relative mobility of 0.26. From the position of this band relative to standard proteins (M_r ranging from 20 100 to 94 000), the subunit M_r was estimated to be 63 000 ± 2000. Gel filtration on a Protein Pak 300SW FPLC column with several standard proteins gave an M_r for native enzyme of 250 000 ± 5000. Thus, isocitrate lyase from *C. acremonium* like the enzyme from other sources (Vanni et al., 1990) is a tetramer of identical or similar subunits. The Stokes radius of the enzyme calculated as described by Siegel and Monty (1966) was 5.3 nm. From this value, we have estimated for the diffusion coefficient ($D_{20,w}$) a value of 4.06×10^{-7} cm^2 s^{-1}. The values of both parameters are close to those described for the enzymes from several sources [see for a review Vanni et al. (1990)] and from *P. blakesleeanus* (Rúa et al., 1990).

The activity of *C. acremonium* isocitrate lyase was very unstable, like that of most isocitrate lyases (Vanni et al., 1990). This problem was overcome by dialyzing for 24 h against 1000 mL of 50 mM Mops/NaOH, pH 7.3, containing 1 mM EDTA, 1 mM benzamidine hydrochloride, 1 mM dithiothreitol, 1.2 mM PMSF, and 40% glycerol. Under these conditions, the activity was found to be stable for 2 months at −20 °C.

We have obtained two apparent activation energy (E_a) values for isocitrate cleavage catalyzed by the *C. acremonium* enzyme (data not shown). The apparent activation energy (E_{a1}) at lower temperatures (below 37.5 °C) was 72.3 kJ mol^{-1}, which is similar to that reported for the thermophilic *Bacillus* enzyme (Chell et al., 1978), but higher than that reported for the *P. indigofera* enzyme (Rao & McFadden, 1965) and for the *Phycomyces* enzyme (Rúa et al., 1990). The apparent activation energy (E_{a2}) at the higher temperatures was 4.7 kJ mol^{-1}, 37.5 °C being the transition temperature. For the *Phycomyces* enzyme, we had reported a similar value for the transition temperature but with a slightly higher apparent activation energy, E_{a2} (Rúa et al., 1990). The *C. acremonium* isocitrate lyase was inactivated by temperature, with first-order kinetics at any temperature (35, 40, and 42.5 °C). In the absence of any added protector, the *C. acremonium* isocitrate lyase retains 80% activity after being heated at 40 °C for 5 min. The temperature for thermal inactivation of isocitrate lyase varies notably from one source to another, and the stabilizer effect of protectors is different (Vanni et al., 1990). From Arrhenius plots of inactivation rate constants [log k *vs* $1/T$ and log (k/T) *vs* $1/T$], we have calculated the activation energy, enthalpy, and entropy for the thermal inactivation of 375 ± 19 kJ mol^{-1}, 370 ± 19 kJ mol^{-1}, and 0.88 ± 0.04 kJ mol^{-1} K^{-1}, respectively. These values for thermodynamic parameters are in the normal range for thermal denaturation. Since $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ values are not significantly affected by temperature, thermal inactivation only resulted from an enhanced entropic contribution to $\Delta G^{\ddagger}$, which in turn reflected the increased structural fluctuations of the polypeptide chain.

pH Dependence of Isocitrate Lyase. The optimum pH for activity of the cleavage reaction was between pH 6.5 and 7.0 in 25 mM imidazole hydrochloride buffer, whereas for the condensation (reverse) reaction the pH optimum was between pH 7.5 and 8.0, in good agreement with that described for isocitrate lyases from other sources (Vanni et al., 1990).

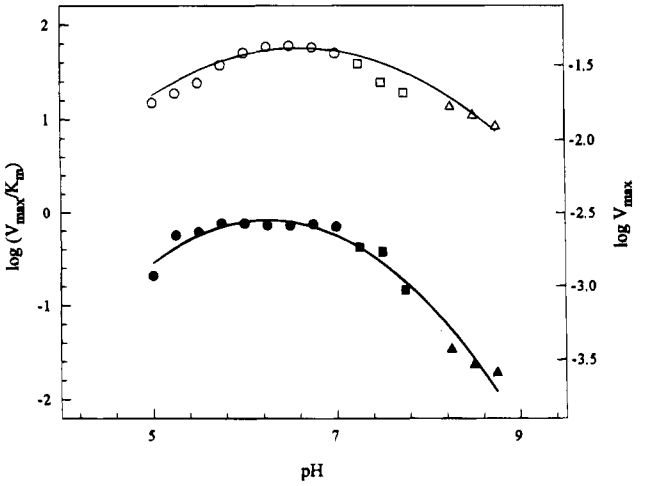

FIGURE 1: Variation with pH of log $V_{max,pH}$ (open symbols) and log($V_{max,pH}/K_{m,pH}$) (solid symbols) for isocitrate cleavage at 30 °C catalyzed by isocitrate lyase from *C. acremonium*. The buffers used were 25 mM Mes/NaOH (●, ○), 25 mM Mops/NaOH (■, □), and 25 mM Tris-HCl (▲, △).

The effect of pH on the cleavage reaction catalyzed by isocitrate lyase was determined by varying the Mg^{2+}–isocitrate concentration at any given pH. We use the following buffers: 50 mM Mes/NaOH (pH 5.0–6.3), 50 mM Mops/NaOH (pH 6.5–7.7), and 50 mM Tris-HCl (pH 8.0–9.0). At each pH value, we obtained values of K_m and V_{max} by plotting the data as double-reciprocal plots as described under Experimental Procedures.

Figure 1 shows the pH dependence of kinetic parameters in the reaction catalyzed by isocitrate lyase. The V_{max} profile is a bell-shaped curve with limiting slopes of +1 on the acid side and −1 on the alkaline side. From this figure, we conclude that the catalysis was dependent on two dissociable groups on the enzyme–substrate complex. Fitting the data to eq 1 yielded values of 5.2 ± 0.1 for pK_A and 7.9 ± 0.3 for pK_B. The V_{max}/K_m profile is also a bell-shaped curve with limiting slopes of +1 on the acid side and −1 on the alkaline side. The data fitted to eq 2 yielded values of 5.2 ± 0.1 for pK_A and 7.6 ± 0.4 for pK_B. In addition, we calculated the standard enthalpy of ionization ($\Delta H°_{ion}$) for the acid and basic groups of the V_{max} profile. These values were obtained from the slopes of plots of pK_A *vs* $1/T$ and pK_B *vs* $1/T$ according to eq 6:

$$-\log K_{eq} = \frac{\Delta H°}{2.3R}\frac{1}{T} - \frac{\Delta S°}{2.3R} \qquad (6)$$

We obtained the same value for $\Delta H°$ of 31.5 kJ/mol for the acid and the basic group. The fact that the pK values for both acid and alkaline sides were similar for the $V_{max,pH}$ and $V_{max,pH}/K_{m,pH}$ profiles indicates that two ionizing groups with pK values of 5.2 and 7.6–7.9, respectively, are involved in catalysis, but probably not in the binding of the Mg^{2+}–isocitrate complex. The pK and $\Delta H°$ values for the ionizing group on the acid side together with the results of diethyl pyrocarbonate modification described below are consistent with a catalytic role for a cationic acid group which may well be the imidazole group of a histidine residue. The proposed catalytic mechanism for isocitrate lyase (Vanni et al., 1990) involves acid–base catalysis, assigning a catalytic role to a histidyl residue, consistent with pH–activity curves for *Neurospora* and *Pseudomonas* enzymes (Rogers & McFadden, 1976, 1977). We also proposed the existence of a histidine residue involved in the catalysis of the *Phycomyces* enzyme (data to be published elsewhere), and recently Diehl and McFadden (1994) have reported that histidine 197 is critical for *E. coli* isocitrate lyase activity and is involved in the catalytic mechanism.

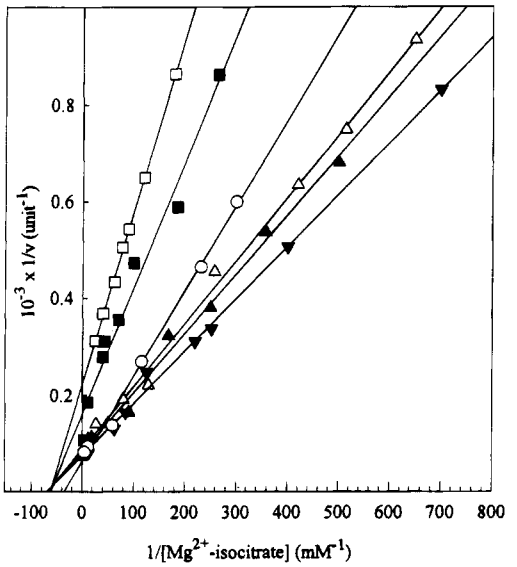

FIGURE 2: Double-reciprocal plot of v^{-1} *versus* 1/[Mg^{2+}–isocitrate] at different fixed free Mg^{2+} concentrations. The free Mg^{2+} concentrations used were 0.02 (□), 0.1 (■), 0.5 (△), 1 (▲), 3 (▼), and 5 mM (○). The reactions were carried out in 25 mM imidazole hydrochloride buffer, pH 6.8, at 30 °C.

On the other hand, the pK and $\Delta H°$ values found by us for the ionizing group on the alkaline side suggest that a cysteine group may also be involved in *C. acremonium* isocitrate lyase catalysis. Chemical modification studies have pointed out the importance of cysteine residues in isocitrate lyases from different sources (Nimmo et al., 1989; Olano et al., 1992). The activity of isocitrate lyase could be restored by treating the inactivated enzyme with 50 mM DTT for 10 min (data not shown). Diehl and McFadden (1994) suggest, for the *E. coli* isocitrate lyase, that the conserved Cys 195 may play a plausible role with His 197 in enhancing catalysis.

Role of Mg^{2+} Ions and Kinetics. Isocitrate lyase from *C. acremonium* has an essential Mg^{2+} requirement for catalysis, but at Mg^{2+} concentrations higher than 5 mM exerts an inhibitory effect. Mg^{2+} can be partially replaced by Mn^{2+} and Ca^{2+}, yielding 75% and 18%, respectively, of the activity with Mg^{2+}. A similar behavior with respect to Mg^{2+} has been reported for all other isocitrate lyases studied (Vanni et al., 1990). However, few attempts have been made to clarify the Mg^{2+} role in isocitrate lyase kinetics. Recently, Giachetti et al. (1988) and Giachetti and Vanni (1991), from detailed kinetic studies on *Pinus pinea* isocitrate lyase, have demonstrated, assuming rapid-equilibrium conditions, that the true substrate for the enzymatic reaction is the Mg^{2+}–isocitrate complex and that Mg^{2+} acts as a nonessential activator. We have also described a similar behavior for isocitrate lyase from *P. blakesleeanus* (Rúa et al., 1990). The double-reciprocal plots of v^{-1} *vs* [Mg^{2+}–isocitrate]$^{-1}$ complex concentrations at different fixed free Mg^{2+} concentrations (Figure 2) showed that the lines have no common intersection point, this pattern being consistent with a mixed-type activation model as proposed by Giachetti et al. (1988). $V_{max_{app}}$ and $K_{m_{app}}$ increase as free Mg^{2+} concentration in-

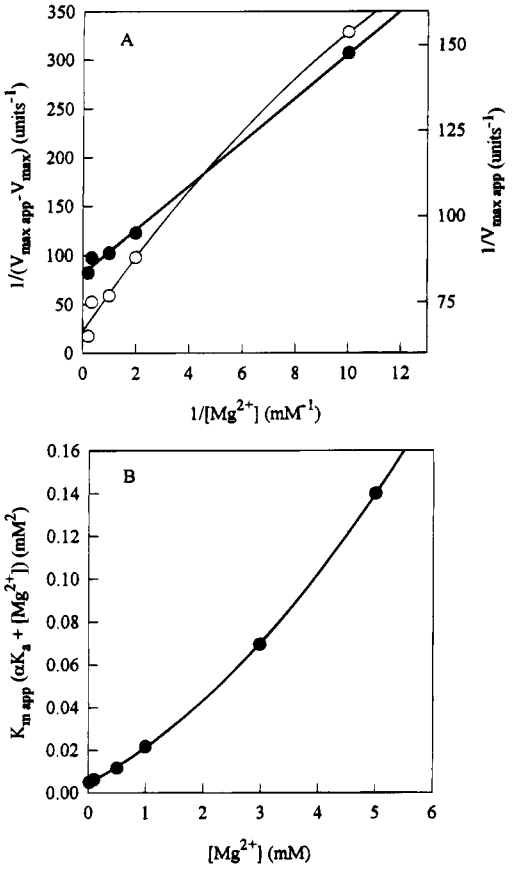

FIGURE 3: Secondary replots of $V_{max_{app}}$ and $K_{m_{app}}$. (A) Replot of $1/V_{max_{app}} - V_{max}$ *versus* $1/[Mg^{2+}]$ (●) from eq 3 fits a linear regression. V_{max} was evaluated as the value for which the correlation coefficient of the linear regression had the highest absolute value. $1/V_{max_{app}}$ *versus* $1/[Mg^{2+}]$ fits a quadratic regression (○). (B) Replot of $k_{m_{app}}(\alpha K_a + [Mg^{2+}])$ *versus* $[Mg^{2+}]$ from eq 4 fits a quadratic regression.

creases. This model assumes that Mg^{2+} binds to an activator site and also to the catalytic site on the enzyme. Binding of Mg^{2+} to the activator site is an obligatory step for the binding of Mg^{2+} to the catalytic site, but the ternary complex AEA is a dead-end form. Mg^{2+}−isocitrate complexes bind to both the activated and the nonactivated enzyme forms, and both enzyme−substrate complexes are catalytically active. The model also includes the binding of free isocitrate to the catalytic site of the nonactivated enzyme, but Giachetti et al. (1988) did not take this into account, because the K_s was much higher than the K_m for substrate, so no physiological significance may be attributed to it. We have not taken into consideration this binding to *C. acremonium* isocitrate lyase since (i) we did not find substrate inhibition in the isocitrate concentration range studied and (ii), as will be described from protection experiments by free isocitrate of DEP modification, the K_s obtained was 6.3 mM, whereas the K_m for substrate (the Mg^{2+}−isocitrate complex) was 0.038 mM, giving a ratio of 165. Thus, we have analyzed our results using the graphical approach described by Giachetti and Vanni (1991). Figure 3 shows the secondary replots of these apparent parameters as a function of $[Mg^{2+}]$. The replot of $1/(V_{max_{app}} - V_{max})$ *versus* $1/[Mg^{2+}]$ fits to a quadratic (r^2 = 0.992) or to a linear regression (r^2 = 0.990). These results support model B6 (Segel, 1975a) and the model of Giachetti et al. (1988). The best fit obtained by applying a quadratic regression to the replot of $K_{m_{app}}(\alpha K_a + [Mg^{2+}])$ *versus* $[Mg^{2+}]$ (r^2 = 1.0), rather than by fitting to a linear regression the same replot (r^2 = 0.994), rejects the B6 activation model (Segel, 1975a) and supports the model of Giachetti et al. (1988). The kinetic constants determined are summarized in Table 2. It is noticeable that both the nonactivated enzyme and the activated enzyme have the same affinity for Mg^{2+}−isocitrate complex (K_{sa} = 15.6 μM, α ≃ 1), whereas the active complex Mg^{2+}−enzyme−[Mg^{2+}−isocitrate] was about 5-fold more (β = 4.83) active catalytically than the active complex enzyme−[Mg^{2+}−isocitrate]. For the *Pinus pinea* enzyme, both active complexes were similarly active (β = 1.58) whereas the affinity of the activated enzyme for the Mg^{2+}−isocitrate complex was 20-fold lower (α = 20) than that of the nonactivated enzyme (Giachetti & Vanni, 1991). On the other hand, the K_a and K'_{ea} values calculated by us as similar to those reported for *Pinus pinea* isocitrate lyase, but K_{sa} is 5-fold higher than that determined for the same enzyme (Giachetti & Vanni, 1991). Our results may suggest that the binding of Mg^{2+} to the activation site (K_a = 0.3 mM) significantly contributes to the catalytic mechanism to enhance catalysis. In order to determine the kinetic mechanism followed by *C. acremonium* isocitrate lyase, we examined the condensation reaction at various concentrations of both glyoxylate (0.0125−4 mM) and succinate (0.125−20 mM). Substrate inhibition by succinate above 8 mM was observed. Linear double-reciprocal plots were obtained when the concentration of either glyoxylate or succinate was varied at fixed concentrations of the other substrate. In both cases, the extrapolated straight lines intersected in the upper-left quadrant. Secondary replots of intercepts and slopes were linear with the reciprocal of concentrations of glyoxylate and succinate (results not shown). This kinetic pattern indicates that the condensation reaction proceeds by a sequential reaction mechanism. On the other hand, with regard to the cleavage reaction catalyzed by the *C. acremonium* isocitrate lyase, double-reciprocal plots of initial velocity *versus* Mg^{2+}−isocitrate concentration in the presence of fixed concentrations of succinate (0.05, 2, 5, 7.5, and 10 mM) gave a family of straight lines intersecting to the left of the ordinate above the abscissa. The replots of slopes and ordinate intercepts *versus* succinate concentration were linear (data not shown). Thus, succinate was a linear mixed-type inhibitor, as expected for an ordered Uni-Bi mechanism (Segel, 1975b) in which glyoxylate binds first to the enzyme, with subsequent binding of succinate. Owing to interference with the assays, inhibition by glyoxylate as the reaction product could not be tested. However, oxalate, an analogue of glyoxylate (Jameel et al., 1985; Rúa et al., 1990; Rogers & McFadden, 1977; Vanni et al., 1990), behaved as a linear competitive inhibitor (K_i = 1.3 μM) with regard to the Mg^{2+}−isocitrate complex, as judged from the kinetic pattern

Table 2: Rate and Dissociation Constants of *C. acremonium* Isocitrate Lyase in the Presence of Mg^{2+}, Calculated Assuming the Activation Mechanism Model Proposed by Giachetti et al. (1988)[a]

constant	value
V_{max} (s^{-1})	4.44 ± 0.3
β	4.83 ± 0.01
α	0.93 ± 0.05
K_{sa} (μM)	15.6 ± 0.1
K'_{ea} (mM)	5.7 ± 0.2
K_a (mM)	0.30 ± 0.01

[a] The values of parameters were determined by fitting the experimental data to eqs 3 and 4. The V_{max} value was expressed as the molecular activity of the enzyme, taking 250 000 as the M_r.

Table 3: Kinetic and Rate Constants of Isocitrate Lyase from *C. acremonium*[a]

$K_{m,A}$ (mM)	0.038	k_{+1} (M^{-1} s^{-1})	244500
$K_{m,P}$ (mM)	0.4	k_{-1} (s^{-1})	15.7
$K_{m,Q}$ (mM)	0.09	k_{+2} (s^{-1})	57.5
K_{ia} (mM)	0.064	k_{-2} (M^{-1} s^{-1})	183000
K_{iq} (mM)	0.17	k_{+3} (s^{-1})	29.5
K'_{ip} (mM)	1.15	k_{-3} (M^{-1} s^{-1})	174200
K_{ip} (mM)	1.73	K_{eq} (M^{-1})	2630
$V_{max,f}$[b] (s^{-1})	19.5 (pH 6.8)		
$V_{max,r}$[c] (s^{-1})	15.7 (pH 7.5)		

[a] Abbreviations: A, Mg^{2+}–isocitrate; P, succinate; Q, glyoxylate; K_m, Michaelis constants; K_i, inhibition constants; K_{eq}, equilibrium constant, mean from the two Haldane equations (Segel, 1975b); $V_{max,f}$ and $V_{max,r}$, V_{max} of the cleavage and the condensation reaction, respectively. The condensation and cleavage reactions were performed at 30 °C in the presence of 5 mM free Mg^{2+}. [b] Based on a M_r of 250 000.

obtained from double-reciprocal plots of initial velocity *vesus* Mg^{2+}–isocitrate concentration at fixed oxalate concentrations and from the linearity of the replots of slopes and the reciprocal of abscissa intercepts *versus* oxalate concentration (data not shown). These results support an ordered Uni-Bi mechanism as proposed for the *E. coli* enzyme (Ko & McFadden, 1990b) and for most isocitrate lyases studied (Vanni et al., 1990). However, a random mechanism has been reported for the *Ricinus* enzyme (Malhotra et al., 1984) and at higher concentrations of succinate or glyoxylate for the *P. indigofera* isocitrate lyase (Williams et al., 1971) and for the *N. crassa* enzyme (Johanson et al., 1974). We have also reported for the *Phycomyces* enzyme (Rúa et al., 1990) that the kinetic mechanism could be preferentially ordered. Thus, although under standard kinetic conditions the kinetic mechanism is practically ordered, probably isocitrate lyase should be considered to have a random kinetic mechanism with a highly preferred pathway, as has been pointed out by Vanni et al. (1990). Table 3 summarizes the kinetic and rate constants for *C. acremonium* isocitrate lyase on the basis of an ordered Uni-Bi mechanism. The K_m value for the Mg^{2+}–isocitrate complex (38 μM) estimated from this kinetic study (at 5 mM free Mg^{2+}) is close to that calculated by us from the analysis of the Mg^{2+} activation model. The values of the other kinetic constants are in the range reported for the isocitrate lyase from *Pinus pinea* (Pinzauti et al., 1982), *Lupinus alba* (Vincenzini et al., 1986), and *Phycomyces* (Rúa et al., 1990). From the estimated values for the individual reaction rate constants, it seemed reasonable to us to suppose that the dissociation of glyoxylate from the enzyme–glyoxylate complex (condensation reaction) would be the limiting step in each direction of the reaction catalyzed by isocitrate lyase from *C. acremonium*. For the reaction in the direction of isocitrate formation, we have determined a value for the equilibrium constant of 2630 M^{-1} (pH 7.5, 30 °C, $\Delta G°$ = −4.7 kcal/mol) according to Haldanes' relationships (Segel, 1975b). Comparable results have been obtained by us for the *P. blakesleeanus* enzyme (K_{eq} = 1400 M^{-1}, pH 7.5, 30 °C, $\Delta G°$ = −4.34 kcal/mol) and for the *Pinus* enzyme (K_{eq} = 937 M^{-1}, pH 7.0, 30 °C, $\Delta G°$ = −4.1 kcal/mol) (Pinzauti et al., 1982) and for *Lupinus* isocitrate lyase (K_{eq} = 944 M^{-1}, pH 7.0, 30 °C, $\Delta G°$ = −4.1 kcal/mol) (Vincenzini et al., 1986).

Inactivation of C. acremonium by Diethyl Pyrocarbonate. Role of Histidine Residues. Isocitrate lyase shows a time-dependent DEP inactivation in Mops/NaOH buffer, pH 7.3, containing 1 mM EDTA. Since DEP is hydrolyzed in aqueous solutions (the half-life is dependent on pH, temperature, and composition and concentration of the buffer), the fraction of remaining activity (A/A_0) should be corrected for decomposition of DEP using the equation (Gomi & Fujioka, 1983):

$$\ln(A/A_0) = -(k/k')I_0(1 - e^{k't})$$

where I_0 is the initial concentration of DEP, k is the second-order rate constant for the reaction of the enzyme with the reagent, and k' is the first-order rate constant for hydrolysis of the reagent. In Mops/NaOH buffer, pH 7.3 and 20 °C, k' was estimated to be 11.22×10^{-2} min^{-1}. Enzyme inactivation at all DEP concentrations used approximates to first-order kinetics, since the plots of $\ln(A/A_0)$ *versus* $(1 - e^{-k't})/k'$ are linear. The pseudo-first-order rate of inactivation (k_{app}) for each DEP concentration, deduced from the slope of inactivation kinetics, shows an apparent saturation effect, as was reported by Saluja and McFadden (1980), although nonsaturating inactivation kinetics had also been described (Jameel et al., 1985). Thus, the reaction of isocitrate lyase from *C. acremonium* with DEP appears to proceed through the formation of a noncovalent enzyme–DEP complex, according to the scheme:

$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} [E{\cdot}I] \xrightarrow{k_{+2}} E{-}I$$

From a plot of $1/k_{app}$ *vs* 1/[DEP], the values of both K_I (the dissociation constant for the enzyme–inactivator complex) and k_2 (the first-order rate constant of inactivation of the enzyme–inactivator complex) were calculated to be 0.56 mM and 0.86 min^{-1}, respectively, at pH 7.3. The second-order rate constant (k_2/K_I) was 25.77 M^{-1} s^{-1}. The pseudo-first-order rate constant (k_{app}) increases with a rise (5.8–7.7) in pH, with a value for k_{max} of 0.13 min^{-1} and a pK_a = 6.0. These values were calculated from the intercept and the slope, respectively, of the plot of $1/k_{app}$ *vs* $[H^+]$ according to the equation by Burnstein et al. (1974):

$$k_{app} = \frac{k_{max}}{1 + [H^+]/K_a}$$

As DEP reacts only with the deprotonated form of imidazole and with the imidazole ring of histidine residues in proteins (Holbrook & Ingram, 1973), and since the pK of 6.0 is characteristic for a histidine residue, we assumed that the inactivation of *C. acremonium* isocitrate lyase by DEP is a consequence of the modification of a histidine residue. Similar pK values have been reported for the modified histidine residues in other proteins (Cousineau & Meighen, 1976; Lundblad & Noyes, 1984; Malhotra & Singh, 1992). The second-order rate constant (k_2/K_I) for the inactivation of isocitrate lyase by DEP at pH 7.3 of 25.77 M^{-1} s^{-1} is similar to the ones reported for other enzymes (Ko et al., 1991; Rúa et al., 1992). The presence of one or more reactive histidine residues at or near the active site of isocitrate lyase can be supported both by the saturation kinetics of the inactivation reaction and by the value of that second-order rate constant. In addition, we examined the difference spectrum (290–220 nm) of the DEP-modified and the native *C. acremonium* isocitrate lyase. Figure 4 shows only one peak with an

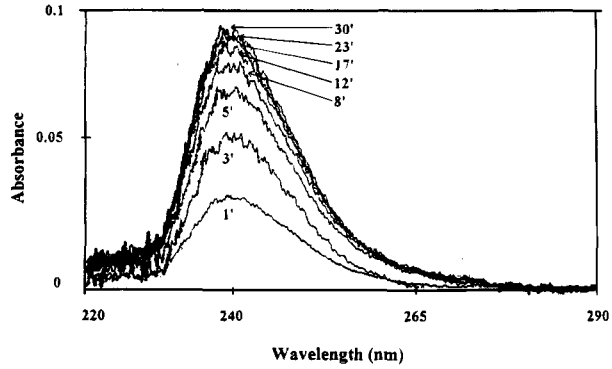

FIGURE 4: UV difference spectra showing the effect of incubation time of *C. acremonium* isocitrate lyase with DEP. The experiment was carried out in 50 mM Mops/NaOH buffer, pH 7.3 at 20 °C, with 0.25 mM DEP and isocitrate lyase at 2.44 μM subunits. The incubation times were 1, 3, 5, 8, 12, 17, 23, and 30 min. The absorbance shown was corrected for blank isocitrate lyase in the range 220−290 nm for each period of incubation.

absorption maximum at 240 nm, whose absorbance increased with the time of modification. This peak and the absence of any change in absorbance at 280 nm support the fact that DEP reacts with histidine residues but not with tyrosine residues (Miles, 1977). We found a linear relationship between the extent of modification of histidine residues calculated from the change in A_{240nm} and the inactivation of isocitrate lyase (Figure 5A). From these data, we may conclude that 11 histidine residues per monomer subunit of the enzyme were modified before complete inactivation. In order to determine how many of these residues are catalytically important, we analyzed our data according to Tsou's (1962) statistical method, which relates the remaining activity A/A_0 with the number of essential residues (i) and with the fraction of residues remaining using the equation:

$$X = (A/A_0)^{1/i}$$

The method is appropriate when all residues are equally reactive toward the modifier reagent (DEP) and on the assumption that modification of any of the essential residues results in complete inactivation. A plot of X *versus* $(A/A_0)^{1/i}$ (Figure 5B) gave a straight line when i equals 1, indicating that 1 histidine residue of the 11 found is catalytically important. The plot with the experimental data did not give straight lines for any value of i other than 1. In a similar way were analyzed the DEP modification data of watermelon cotyledons isocitrate lyase (Jameel et al., 1985). They find that only one histidine residue of the five modified is essential.

On the other hand, after hydroxylamine treatment (Figure 6), the incremental absorption at 242 nm characteristic of the *N*-carbethoxyhistidine residues in the enzyme (Miles, 1977) was reversed almost completely, the activity returning to approximately 80% from the 20% of the DEP-modified enzyme (with 0.5 mM DEP; see Experimental Procedures for details). This restoration of the activity indicates that no excess of modifier was used and that the inactivation did not result from denaturation of the enzyme. Moreover, the fast decrease in absorbance at 240 nm after hydroxylamine treatment is representative of histidine modification, while on the contrary, the decarbethoxylation of *O*-(ethoxyformyl)-tyrosyl derivatives is known to occur at a much slower rate (Melchior & Fahrney, 1970). When the enzyme was

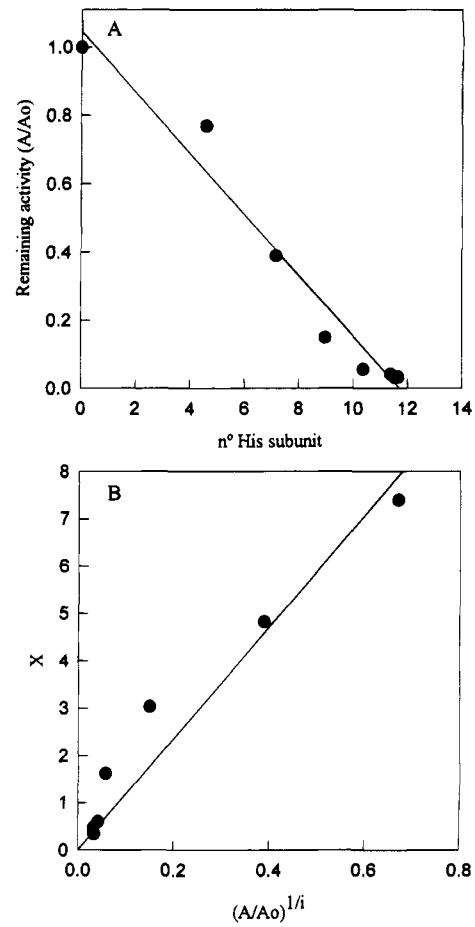

FIGURE 5: Number of histidine residues modified and the residual enzyme activity. The enzyme sample (2.44 μM subunits) was inactivated with 0.25 mM DEP as described under Experimental Procedures, at pH 7.3 in 50 mM Mops buffer. Aliquots were removed in intervals and assayed for enzyme activity. The number of histidines modified was calculated from the increase in absorbance at 240 nm by using a difference absorbance of 3.2×10^3 M^{-1} cm^{-1}. (A) Plot of the residual enzyme activity against the number of residues modified. (B) Tsou's analysis of the data.

inactivated with a higher concentration of DEP (1 mM), the activity recorded was only 50−60% from a residual activity of 10%. Recovery of 80−100% of watermelon cotyledons isocitrate lyase activity depending upon the extent of DEP inactivation has been reported (Jameel et al., 1985). DEP can also react with serine, arginine, cysteine, and lysine (Mühlrád et al., 1967; Melchior & Fahrney, 1970; Avaeva & Krasnova, 1975; Miles, 1977; Horiike et al., 1979), but the modification can be restored by hydroxylamine in all cases except when produced by lysine (Miles, 1977; Saluja & McFadden, 1980).

The possibility that Cys residues may have reacted with DEP should be ruled out for two reasons: (i) we have not detected any increase at 230 nm, which is the absorption maximum wavelength for *N*-acetylcysteine modified with DEP (Saluja & McFadden, 1980); and (ii) since *C. acremonium* isocitrate lyase can be rapidly and completely inactivated by the thiol reactive reagent 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and afterward fully reversed by treatment with dithiothreitol (DTT) (data not shown), we carried out the next experiment with this modifier. Isocitrate lyase (2.5 μM subunit concentration) was incubated with DTNB (1 mM), and when no more activity was noted, (after 10 min incubation), DEP (0.5 mM) was added to the

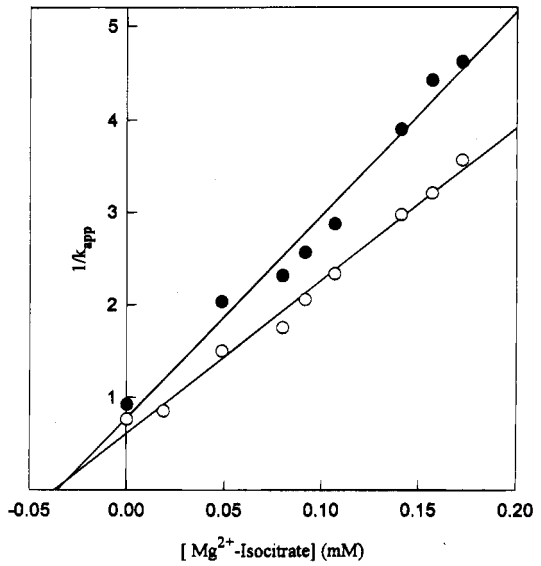

FIGURE 6: Effect of hydroxylamine on DEP-inactivated isocitrate lyase. The enzyme (2.44 μM subunits) was inactivated with 0.25 mM DEP in 50 mM Mops buffer, pH 7.3 for 30 min, after which 0.5 M hydroxylamine (final concentration) was added. The difference in A_{240nm} between the DEP-treated enzyme and the control (isocitrate lyase alone) was registered. The reagent was added to both cuvettes.

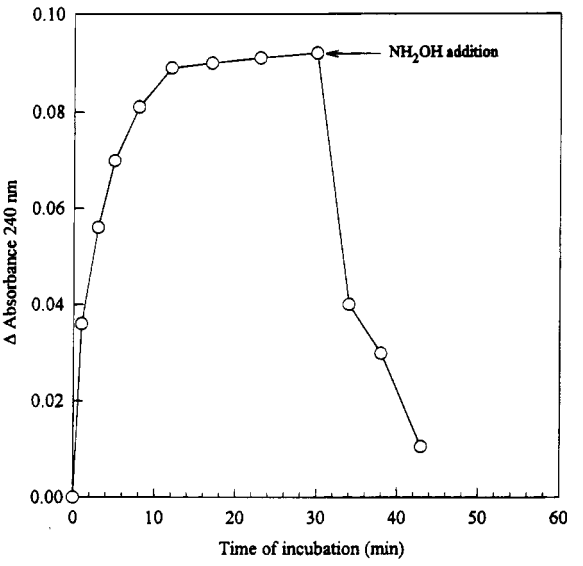

FIGURE 7: Protection by Mg^{2+}−isocitrate complex against inactivation of *C. acremonium* isocitrate lyase by DEP. Determination of the K_s for the Mg^{2+}−isocitrate complex. The protective data (k_{app} values) were obtained as indicated in the text from the slopes of a plot of ln([P_∞] − [P]) *versus* *t*. The DEP concentrations used were (●) 0.5 mM and (○) 1 mM.

incubation mixture, and after a further 10 min, histidine (16 mM) was added to quench the excess of DEP. DTT (50 mM) did not produce any recovery of enzyme activity when added to this incubation mixture, but the addition of hydroxylamine (0.5 M) before DTT resulted in a recovery of 50% of the preincubation enzymatic activity. Incubation of isocitrate lyase with DTNB prior to the addition of DEP ensures that Cys residues involved in the catalytic action are blocked and so DEP cannot react with them. The results obtained indicate that the treatment with DEP modifies histidine residues.

Since a catalytic role as proton donor has been assigned to a lysyl residue for the watermelon enzyme (Vanni et al., 1990) and because slower reactions of DEP with lysyl residues may convert the enzyme into a nonreversible enzyme state, we think that the unrecovered enzymatic activity may be due to the modification of a lysyl residues (Miles, 1977).

We tested the effect of D_s-isocitrate, Mg^{2+} ions, and the Mg^{2+}−D_s-isocitrate complex on DEP inactivation of *C. acremonium* isocitrate lyase.

The protective effect of Mg^{2+}−D_s-isocitrate complex was studied in the range of 0.01−0.2 mM and at two fixed concentrations of DEP (0.5 and 1 mM).

Each complex concentration was obtained with 0.2 mM total Mg^{2+}. Protective k_{app} data were obtained according to the equation (Tian & Tsou, 1982):

$$\ln([P_\infty] - [P]) = \ln [P_\infty] - k_{app}[I]t$$

by a plot of ln([P_∞] − [P]) *versus* *t*, where [P_∞] and [P] represent the concentration of product formed at time approaching infinity and at time *t*, respectively, and [I] is the DEP concentration. The Mg^{2+}−D_s-isocitrate complex provides total protection against inactivation of the enzyme by DEP, indicating that the imidazole group modified belongs to a histidyl residue probably present at the active site of the *C. acremonium* isocitrate lyase. The protection data were analyzed by treating the Mg^{2+}−D_s-isocitrate complex as a total competitive inhibitor of the inactivation reaction (Dixon & Webb, 1979). From plots of $1/k_{app}$ *versus* Mg^{2+}−D_s-isocitrate concentrations (Figure 7) for each DEP concentration, we obtained straight lines intersecting in the upper-left quadrant. From the abscissa of the intersection point, we directly calculated a K_s value for Mg^{2+}−D_s-isocitrate of 26.8 ± 2.1 μM, which is in good agreement with the kinetically determined K_m value.

This result indicates that the protection against inactivation is a consequence of binding of Mg^{2+}−isocitrate complex and that modified histidyl residues are at or near the active site of the enzyme.

On the other hand, either D_s-isocitrate in the absence of Mg^{2+} ions or Mg^{2+} ions in the absence of D_s-isocitrate also product total competitive protection against DEP modification. Jameel et al. (1985) had reported that D_s-isocitrate protects one essential histidine residue of watermelon cotyledons isocitrate lyase against DEP modification. From a plot of $1/k_{app}$ *versus* D_s-isocitrate concentrations at two fixed DEP concentrations, and from a plot of $1/k_{app}$ *versus* Mg^{2+} ion concentration at the same two fixed DEP concentrations, we have calculated a K_s for D_s-isocitrate of 6.3 ± 0.15 mM and a K_s for Mg^{2+} ions of 7.5 ± 0.2 mM, respectively.

The total competitive protection by isocitrate suggests the binding of free isocitrate to the catalytic site of the nonactivated enzyme, although without physiological significance, due to the high ratio of its K_s to the K_m for substrate, the Mg^{2+}−isocitrate complex, in agreement with that proposed for the *Pinus pinea* enzyme (Giachetti et al., 1988).

The K_s for Mg^{2+} ions obtained from protection experiments, when compared with the dissociation constants for Mg^{2+} reported in Table 2, is consistent with the fact that Mg^{2+} ion protection arises from its binding to the catalytic site, since at the Mg^{2+} ion concentrations used the enzyme will be in the activated form according to the model analyzed (Giachetti et al., 1988; Giachetti & Vanni, 1991).

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