Evidence for an essential arginine residue at the active site of ATP citrate lyase from rat liver

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Rat liver ATP citrate lyase was inactivated by 2,3-butanedione and phenylglyoxal. Phenylglyoxal caused the most rapid and complete inactivation of enzyme activity in 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid buffer, pH 8. Inactivation by both butanedione and phenylglyoxal was concentration-dependent and followed pseudo-first-order kinetics. Phenylglyoxal also decreased autophosphorylation (catalytic phosphate) of ATP citrate lyase. Inactivation by phenylglyoxal and butanedione was due to the modification of enzyme arginine residues; the modified enzyme failed to bind to CoA-agarose. The V declined as a function of inactivation, but the K_m values were unaltered. The substrates, CoASH and CoASH plus citrate, protected the enzyme significantly against inactivation, but ATP provided little protection. Inactivation with excess reagent modified about eight arginine residues per monomer of enzyme. Citrate, CoASH and ATP protected two to three arginine residues from modification by phenylglyoxal. Analysis of the data by statistical methods suggested that the inactivation was due to modification of one essential arginine residue per monomer of lyase, which was modified 1.5 times more rapidly than were the other arginine residues. Our results suggest that this essential arginine residue is at the CoASH binding site.

ATP citrate lyase [ATP citrate (*pro-3S*)-lyase; EC 4.1.3.8] is widely distributed in animal tissues and has been purified to homogeneity from rat liver (Inoue *et al.*, 1966; Linn & Srere, 1979) and adipose tissue (Ramakrishna & Benjamin, 1978, 1979a). ATP citrate lyase has a molecular weight of 440 000–450 000 and consists of four identical subunits (M_r 110 000) (Singh *et al.*, 1976). The enzyme is known to be a phosphoprotein with 2 mol of structural phosphate/mol of tetramer. When the enzyme is incubated with ATP, 2 mol of phosphate are covalently linked to a histidine residue/mol of protein (Cottam & Srere, 1969; Ramakrishna & Benjamin, 1979a). The phosphoenzyme can react with Mg-citrate, forming a citryl enzyme which reacts with CoASH to form acetyl-CoA and oxaloacetate. ADP and P_i are released at any of the intermediate steps.

Though the mechanism of this enzyme reaction has been studied in great detail, little is known about the structure of the catalytic site. Enzyme arginine residues are known to play an important role in

binding of anionic substrate(s), cofactor(s) or effector(s) (Riordan *et al.*, 1977). The role of arginine residues in enzymatic activity can be studied by using specific arginine modifiers such as phenylglyoxal and 2,3-butanedione. Enzymes may be protected from inactivation by dicarbonyl reagents by the presence of substrates that bind at arginine residues. Ribulose biphosphate carboxylase is protected by ribulose biphosphate (Schloss *et al.*, 1978), NADPH-dependent aldehyde reductase is protected by NADPH (Davidson & Flynn, 1979), and pyruvate kinase is protected by ATP and phosphoenolpyruvate (Berghauer, 1977). In preliminary studies (Ramakrishna & Benjamin, 1979b) we found that the phosphorylation of an adipose tissue phosphoprotein, which we later identified as ATP citrate lyase (Ramakrishna & Benjamin, 1979a), was markedly inhibited by phenylglyoxal.

Materials and methods

Materials

DEAE-cellulose (DE-52) was purchased from Whatman. Sephadex G-25 (fine) was obtained from Pharmacia. 1,2-Cyclohexanedione and 2,3-butane-

Abbreviation used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

dione were obtained from Aldrich. Tris base, 2-mercaptoethanol, dithiothreitol, CoASH, ATP, ADP, NADH, malic dehydrogenase, hydroxylamine hydrochloride, phenylglyoxal and bovine serum albumin were purchased from Sigma. [γ - 32 P]ATP (2–5 Ci/mmol) was prepared as described by Glynn & Chappell (1964). [14 C]Phenylglyoxal (28 mCi/mmol) was purchased from Research Products International. Agarose–hexane–CoA (type 1) was a product of P-L Biochemicals. All other chemicals used were of analytical reagent grade.

Enzyme assays

When phenylglyoxal was used for modification of ATP citrate lyase, activity was assayed by the hydroxamate method (Takeda *et al.*, 1969), because of the possibility of inhibition of coupling enzyme in the malic dehydrogenase-coupled assay by phenylglyoxal carried over with the sample. The assay mixture contained 200 μ mol of Tris/HCl buffer, pH 8.7, 10 μ mol of dithiothreitol, 20 μ mol of potassium citrate, 10 μ mol of MgSO_4 , 0.33 μ mol of CoASH, 100 μ mol of hydroxylamine and 5 μ mol of ATP in a final volume of 1 ml. Assays were carried out at 37°C. Reactions were terminated by the addition of 1.5 ml of 20% (w/v) trichloroacetic acid/0.4 M- FeCl_3 . The acetylhydroxamate formed was measured by its absorbance at 520 nm. One unit of enzyme activity is defined as the amount of protein necessary to catalyse the formation of 1 μ mol of acetylhydroxamate/min or the amount necessary to catalyze the oxidation of 1 μ mol of NADH/min. Phosphate incorporation was assayed by the phenol-extraction method as described previously (Ramakrishna & Benjamin, 1979a).

Purification of ATP citrate lyase

ATP citrate lyase was purified from rat liver by a modification of the procedure of Linn & Srere (1979). In the DE-52 chromatography step, protein was eluted with a linear gradient of 0.02–0.20 M-potassium phosphate, pH 7.5. Fractions with most ATP citrate lyase activity were pooled, precipitated with $(\text{NH}_4)_2\text{SO}_4$ (35 g/100 ml) and the precipitate was dissolved in buffer A [0.02 M-Tris/HCl, pH 7.2, containing 3 mM-dithiothreitol, 0.1 mM-EDTA, 1 mM- MgSO_4 and 10% (v/v) glycerol] and dialysed overnight against 50 vol. of the same buffer. The dialysed DE-52 fraction was chromatographed on CoA–agarose (column 1 cm $\times$ 20 cm) equilibrated with the same buffer and washed until protein was no longer eluted. ATP citrate lyase was eluted with 0.1 M-citrate in buffer A. The enzyme could also be eluted from the affinity gel by 1 mM-CoASH or 0.5 M-NaCl. Fractions containing most of the enzyme were pooled and concentrated by precipitation with $(\text{NH}_4)_2\text{SO}_4$ (35 g/100 ml). The precipitate was dissolved in a minimal volume (protein

concn. 6–10 mg/ml) of buffer A and dialysed overnight against 100 vol. of this buffer. The purified enzyme was homogeneous by the criterium of gel electrophoresis, without any observable proteolysis. The specific activity of the pure enzyme by the malic dehydrogenase-coupled assay method was about 4.95 units/mg of protein at 20°C and 9.1 units/mg of protein at 37°C, which is comparable with that of the preparation of Linn & Srere (1979).

Details of the purification of the rat adipose tissue enzyme will be published elsewhere. Protein was estimated by the method of Lowry *et al.* (1951), by the dye-binding method (Bradford, 1976) or by its absorbance at 279 nm ($A_{1\text{cm}}^{1\%} = 11.4$).

Reaction with arginine-modifying reagents

Unless otherwise stated, ATP citrate lyase (150–300 μ g/ml) was incubated at 30°C with either 2,3-butanedione in 50 mM-borate buffer, pH 8.0, or phenylglyoxal in 50 mM-Hepes buffer, pH 8.0. Enzyme incubated with buffer alone served as the untreated control. At the time points indicated, samples (100 μ l) were removed and assayed for enzyme activity. In experiments to determine the optimum pH and buffer conditions to produce maximal effects, borate buffer was replaced by Tris/HCl or potassium phosphate buffers at various pH values. Chemical modifiers were prepared fresh daily.

Protection studies

The ability of various ligands to protect ATP citrate lyase against inactivation by butanedione or phenylglyoxal was tested by preincubation of the enzyme (30°C for 5 min) with the ligand followed by incubation with the modifier. Samples were removed at periodic intervals and assayed for enzymic activity.

Kinetic studies of the modified enzyme

Protein incubated with buffer alone (control), with 3 mM-phenylglyoxal for 10 min or 30 min, or preincubated with 5 mM-citrate plus 0.25 mM-CoASH for 5 min followed by 30 min incubation with 3 mM-phenylglyoxal in 50 mM-Hepes buffer, pH 8.0, was chromatographed on a Sephadex G-25 (fine) column (1 cm $\times$ 20 cm) to remove the unreacted reagent. Protein was eluted from the column with buffer A containing 100 mM-NaCl. Fractions (0.75 ml) containing enzyme were pooled and the protein concentrations were made similar by appropriate dilution. The time required from the application of the sample on the column to recovery of the protein was approx. 12 min during which no additional enzyme inactivation was detected. Kinetic parameters of these gel-filtered protein samples were determined by assaying ATP citrate lyase activity by the coupled malic dehydrogenase method.

Affinity chromatography of modified enzyme

Enzyme (1mg/ml) was incubated with 5mM-phenylglyoxal for approx. 40min. This treatment decreased enzyme activity by more than 95%. This sample was first chromatographed on Sephadex G-25 and then loaded at room temperature onto CoA-agarose (1ml column volume). The column was eluted with buffer A, collecting 1ml fractions. After 12 fractions, the column was eluted with 0.1M-citrate in buffer A. An untreated protein sample, processed identically, served as control.

Incorporation of [^{14}C]phenylglyoxal

Radiolabelling of the enzyme by phenylglyoxal was studied by incubating the protein (1.1mg/ml) for various times with [^{14}C]phenylglyoxal (1.5mM, 6116 c.p.m./nmol) in 50mM-Hepes buffer, pH 8.0, at 30°C. At different times, aliquots (100 μl) were pipetted onto 2.5cm² Whatman 3MM filter disks which were immediately dipped in cold 10% (w/v) trichloroacetic acid. The filter disks were washed four times with 10% (w/v) trichloroacetic acid followed by washes with ethanol and diethyl ether. Disks were air-dried and placed in a toluene-based scintillation mixture [0.5% 2,5-diphenyloxazole/0.03% 1,4-bis(5-phenyloxazol-2-yl)benzene] and counted in a LKB 1210 Ultrabeta scintillation spectrometer.

Amino acid analysis

Native and phenylglyoxal-modified enzyme samples (200 μl ; 1.1 mg of protein/ml) were diluted with 40 vol. of 6M-HCl containing 0.1M-2-mercaptoethanol and hydrolysed at 110°C for 24 h. Since the neutral and acidic amino acids were not modified by phenylglyoxal treatment, the hydrolysates were chromatographed on the short column of a Beckman amino acid analyzer.

Results

Inactivation of ATP citrate lyase by phenylglyoxal and 2,3-butanedione

Rat liver ATP citrate lyase was incubated for 10min with 10mM each of phenylglyoxal, 2,3-butanedione, and 1,2-cyclohexanedione in 50mM-borate buffer at pH values 7, 7.5, 8.0, and 8.5. Since 1,2-cyclohexanedione decreased the enzyme activity minimally this reagent was not studied further. Phenylglyoxal and butanedione showed maximal inhibition (85 and 35% respectively) of ATP citrate lyase activity at pH 8.0. Phenylglyoxal was used for most of the studies since it inactivated the enzyme most rapidly.

The logarithm of enzyme activity remaining plotted as a function of time at various phenylglyoxal and butanedione concentrations was linear to less than 10% of the initial enzyme activity (Figs.

1 and 2). Prolonged incubation with phenylglyoxal completely inactivated the enzyme. In controls, enzyme activity remained unchanged when incubated without the inhibitor. Inactivation by these chemical modifiers appeared to follow first-order kinetics at any fixed concentration of phenylglyoxal or butanedione. The reaction order with respect to phenylglyoxal and butanedione concentrations for the inactivation of the lyase was determined from the equation:

$$k_{\text{app.}} = k(M)^n$$

where $k_{\text{app.}}$ is the apparent first-order rate constant for inactivation, k is the second-order rate constant, and n is the reaction order of phenylglyoxal (M) reacting with the enzyme residues to yield an inactive complex. By replotting the data, $\log k_{\text{app.}}$ versus $\log(M)$ (inset of Figs. 1 and 2), a value of 1.0 was obtained for the reaction order (n). Similar inactivation data were observed for ATP citrate

Fig. 1. Inactivation of ATP citrate lyase by phenylglyoxal

ATP citrate lyase (0.3mg/ml) was incubated in 50mM-Hepes buffer, pH 8.0, at 30°C with 1.5mM (○), 3.0mM (△), 6.0mM (□) and 10.0mM (●) phenylglyoxal. At specified times, aliquots (100 μl) were assayed for lyase activity by the hydroxamate method. Protein concentrations were in the linear range of the assay method. Inset: determination of the order of reaction with respect to phenylglyoxal. $-\log(k_{\text{app.}})$ was plotted against $-\log(\text{phenylglyoxal})$ (M). The slope of the line gives the reaction order (n).

lyase from adipose tissue treated with phenylglyoxal. The apparent second-order rate constant (k) for the inactivation of ATP citrate lyase was calculated from the slope of the plot to be

Fig. 2. Inactivation of ATP citrate lyase by butanedione

ATP citrate lyase (0.3 mg/ml) was incubated in 50 mM-borate buffer, pH 8.0, at 30°C with 5 mM (○), 10 mM (△), 20 mM (□), and 30 mM (●) butanedione. The enzyme was incubated with 20 mM-butanedione in 50 mM-Tris/HCl buffer, pH 8.0 (▲), 50 mM-Hepes buffer, pH 8.0 (×), and 50 mM-potassium phosphate buffer, pH 8.0 (▽). Inset: determination of the order of reaction with respect to butanedione in borate buffer. See legend to Fig. 1 for details.

$0.33 \text{ M}^{-1} \cdot \text{s}^{-1}$ for phenylglyoxal in Hepes buffer, pH 8.0, and $0.042 \text{ M}^{-1} \cdot \text{s}^{-1}$ for butanedione in borate buffer, pH 8.0.

Among the various buffers tested at pH 8.0, enzyme inactivation by butanedione (20 mM) (Fig. 2) was highest in borate buffer, followed by potassium phosphate and Hepes buffers, and least in Tris/HCl buffer. Inactivation by phenylglyoxal was maximal in Hepes buffer ($k = 0.33 \text{ M}^{-1} \cdot \text{s}^{-1}$) followed by potassium phosphate buffer ($k = 0.25 \text{ M}^{-1} \cdot \text{s}^{-1}$) and then borate buffer ($k = 0.078 \text{ M}^{-1} \cdot \text{s}^{-1}$) (data not shown). Hence, in all further experiments, inactivation by phenylglyoxal was studied in 50 mM-Hepes buffer, pH 8.0, and the effects of butanedione were studied in 50 mM-borate buffer, pH 8.0.

The first step in the catalytic reaction of ATP citrate lyase is the formation of phosphoenzyme from Mg-ATP (Srere, 1972). Treatment of ATP citrate lyase with phenylglyoxal inhibited enzyme autophosphorylation, which is most likely at the catalytic site (Ramakrishna & Benjamin, 1979b). This inactivation appeared to follow a pseudo-first-order reaction process.

Properties of modified enzyme

The kinetic properties of the enzyme modified by treatment with 3 mM-phenylglyoxal for 10 min and 30 min, and by treatment with 3 mM-phenylglyoxal for 30 min in the presence of 0.25 mM-CoASH and 5 mM-potassium citrate, were investigated (Table 1). The apparent K_m values for ATP, citrate and CoASH were found to be unaltered during the course of inactivation by phenylglyoxal. However, the V of the enzyme decreased in proportion to the inactivation of the enzyme. The pseudo-first-order rate constant for the decrease in V was 0.053 min^{-1} at 3 mM-phenylglyoxal. This value is similar to the k_{app} obtained for inactivation of ATP citrate lyase by 3 mM-phenylglyoxal (Fig. 1). Protection of the enzyme with CoASH plus citrate limited the decrease in V . Inactivation by butanedione also did not alter the K_m values though the rate of decrease in

Table 1. Effect of inactivation by phenylglyoxal on kinetic parameters of ATP citrate lyase
The units for V are millunits (mU) per $4 \mu\text{g}$ protein at 20°C.

Treatment	Kinetic parameters for						
	ATP		Citrate*			CoASH	
	K_m (μM)	V (mU)	K_m (μM)	K_m (mM)	V (mU)	K_m (μM)	V (mU)
Control	144	19.2	117	1.5	18.8	21	18.9
3 mM-Phenylglyoxal (10 min)	161	13.7	122	1.5	13.5	21	13.3
3 mM-Phenylglyoxal (30 min)	155	3.8	108	1.5	3.8	22	4.2
0.25 mM-CoASH + 5 mM-citrate + 3 mM-phenylglyoxal (30 min)	156	8.5	127	1.5	9.1	23	9.1

* Under normal assay conditions a low and a high K_m values are obtained for citrate concentrations.

V values with time was similar to the rate of inactivation (results not shown).

In order to study whether arginine residues were required for substrate binding, enzyme which had

first been inactivated with 5 mM-phenylglyoxal to less than 5% of original activity was chromatographed on Sephadex G-25 to remove unreacted phenylglyoxal. The inactivated enzyme was then chromatographed on CoA-agarose. The modified protein was not retained (Fig. 3), but untreated enzyme was retained on the column and was eluted from the column with 0.1 M-citrate in buffer A. These findings suggest that ATP citrate lyase inactivation by phenylglyoxal may be due to modification of the CoASH binding site.

Protection by substrates against inactivation by phenylglyoxal and butanedione

Inactivation rates produced by these reagents in the presence of various concentrations of enzyme substrates were studied to determine if substrates would protect the enzyme from inactivation by phenylglyoxal and butanedione. Protection studies were performed by preincubating the enzyme with the protector followed by incubation with the modifier. Pseudo-first-order kinetic data were observed for the inactivation of ATP citrate lyase by phenylglyoxal and butanedione in the presence of added ligands (Fig. 4a). The ability of various ligands to protect the lyase from reacting with the modifier is shown in Table 2. Mg-ATP at concentrations lower than 0.4 mM slightly reduced the inactivation produced by α -dicarbonyl reagents.

Fig. 3. *Chromatography of phenylglyoxal-inactivated ATP citrate lyase on CoA-agarose*

Enzyme (1 mg/ml) was inactivated by incubation with 5 mM-phenylglyoxal at 30°C for 40 min in 50 mM-Hepes buffer, pH 8.0, and chromatographed on CoA-agarose as described in the Materials and methods section. Inactivated enzyme (O) and untreated control (●). At the arrow, elution was started with 0.1 M-citrate in buffer.

Fig. 4. *Protection of ATP citrate lyase from inactivation by phenylglyoxal*

(a) Enzyme (0.3 mg/ml) was preincubated for 5 min with 50 mM-Hepes buffer, pH 8.0, (O) or with buffer containing 5 mM-citrate (□), 0.25 mM-CoASH (Δ) and 5 mM-citrate plus 0.25 mM-CoASH (●). Note that the concentrations of protecting agents given are final concentrations after the addition of modifier. During pretreatment the volumes were 3/4 of the final volume after addition of phenylglyoxal. The pretreated enzyme samples were then incubated with 1.5 mM-phenylglyoxal and at specified time points aliquots (100 μ l) were assayed for enzymic activity as described in the legend to Fig. 1 and the Materials and methods section. (b) Kinetic analysis of protection by ligands. Values for k_{app} were obtained from a series of experiments in which the concentration of phenylglyoxal was varied in the presence of fixed concentrations of various ligands. k_{app} was plotted as a function of phenylglyoxal concentration (mM). No added ligand (O), 5 mM-citrate (Δ), 0.25 mM-CoASH (●) and 5 mM-citrate plus 0.25 mM-CoASH (□).

Table 2. *Effect of various ligands on the rate of inactivation of ATP citrate lyase by phenylglyoxal and 2,3-butanedione*
 Values of k_{app} were obtained from the plots of $2.3 \times \log(\text{percentage residual activity})$ against time of incubation with reagent.

Reagent	Ligand	$10^3 \times k_{app} \text{ (min}^{-1}\text{)}$
Phenylglyoxal (5 mM)	—	93.0
	Citrate (2 mM)	52.6
	Citrate (5 mM)	45.2
	Citrate (15 mM)	40.8
	Citrate (40 mM)	37.0
	Mg-ATP (0.4 mM)	67.8
	Mg-ATP (0.8 mM)	108.7
	CoASH (0.25 mM)	24.4
	CoASH (0.5 mM)	24.4
	Citrate (2.0 mM) + Mg-ATP (0.8 mM)	47.1
Butanedione (10 mM)		22.2
	Citrate (5 mM)	10.0
	Citrate (20 mM)	8.3
	Mg-ATP (0.25 mM)	14.8
	CoASH (0.25 mM)	5.9
	CoASH (0.25 mM) + citrate (5 mM)	4.9

However, Mg-ATP at concentrations of 0.5 mM and above did not protect but augmented the inactivation by phenylglyoxal. Citrate (5 mM) decreased the rate of inactivation by 5 mM-phenylglyoxal by approx. 50% and that by 10 mM-butanedione by 65%. With increasing concentrations of citrate (to 40 mM), protection against inactivation by phenylglyoxal increased, but in a non-linear fashion. Citrate plus Mg-ATP did not produce additional protection compared with citrate alone. CoASH (0.25 mM) protected the enzyme from inactivation by approx. 75%. Protection by CoASH plus citrate was higher than when either ligand was added alone (Fig. 4a). ADP, a competitive inhibitor of citrate lyase, did not protect against inactivation by phenylglyoxal. Of the several ligands tested, CoASH appeared to be the most effective in protecting ATP citrate lyase against inactivation by phenylglyoxal or butanedione. Among the substrate combinations tested, citrate plus CoASH gave maximal protection. Inactivation of ATP citrate lyase in the presence of added ligands was also examined at different phenylglyoxal concentrations. Plots of k_{app} as a function of phenylglyoxal concentrations with different ligands were linear (Fig. 4b). The apparent second-order rate constant (calculated from the slope) decreased with protection and the lowest value ($k = 0.13 \text{ M}^{-1} \cdot \text{s}^{-1}$) was observed with citrate plus CoA.

Determination of the number of arginine residues modified

Fig. 5 illustrates the correlation between mol of [^{14}C]phenylglyoxal incorporated into each mol of subunit and the extent of inactivation. The degree of

inactivation during the initial 80–90% loss of enzyme activity was directly proportional to [^{14}C]-phenylglyoxal incorporation. About 17 mol of phenylglyoxal were calculated to be incorporated per monomer of enzyme at 100% inactivation. Studies of the amino-acid composition of the modified enzyme suggested that only arginine residues were modified by phenylglyoxal. A linear relation was also observed between the arginine residues destroyed by phenylglyoxal and loss of enzyme activity (Fig. 5). From these data it was calculated that for every arginine residue modified, approximately 2 mol of phenylglyoxal were incorporated per mol of enzyme.

Radioactive phenylglyoxal incorporation into both the protected and unprotected enzyme was measured to determine the number of arginine residues protected by the ligands. The differences in the extent of incorporation between unprotected and protected enzyme at each time point was plotted against the enzyme activity lost from the unprotected enzyme, according to the method of Schloss *et al.* (1978). A difference of about 4–6 mol of phenylglyoxal incorporated per mol of protected enzyme subunit compared to the unprotected was calculated by extrapolation to 100% inactivation. Thus the ligands (citrate, CoASH, or CoASH plus citrate) protected 2–3 arginine residues per subunit from the total of 8.5 residues modified by phenylglyoxal.

To determine the number of essential arginine residues, the data were analysed by the statistical method of Tsou (1962) as described by Horiike *et al.* (1979). This method requires the determination of both the number of residues modified and the residual enzyme activity in partially modified pro-

Fig. 5. Phenylglyoxal incorporation per monomer of ATP citrate lyase as a function of enzyme activity lost

The enzyme (1.1 mg/ml) was incubated with 1.5 mM- $[^{14}\text{C}]$ phenylglyoxal. Arginines residues modified were determined by amino acid analysis (●); (O), $[^{14}\text{C}]$ phenylglyoxal incorporated into monomer of enzyme. The difference in incorporation between unprotected enzyme and enzyme protected by either 5 mM-citrate (▽), 0.3 mM-ATP (□), 0.25 mM-CoASH (Δ) or 0.25 mM-CoASH plus 0.3 mM-ATP (■) at corresponding time points is given in the lower curves.

tein samples. The data given in Fig. 5 allows such an analysis from the equation:

$$m - n(1 - x) = n - pa^{1/i} - (n - p)a^{\alpha/i} \quad (1)$$

where n is the total number of modifiable arginine residues in the enzyme, among which p of the residues, of which i are essential, react with phenylglyoxal at a pseudo-first order rate constant k_1 and $(n - p)$ residues that are not essential react at a pseudo-first-order rate constant $k_2 (= \alpha k_1)$, a is the fraction of activity remaining, x is the fraction of unmodified arginine residues and m is the number of arginine residues modified per molecule. Eqn. (1) can be written in the following form (Tsou, 1962; Horiike *et al.*, 1979):

$$\log \left[\frac{nx}{a^{1/i}} - p \right] = \log(n - p) + \left(\frac{\alpha - 1}{i} \right) \log a \quad (2)$$

In eqn. (2), the left-hand component was calculated with $n = 8.5$, $p = i = 1$ and plotted against a (Fig. 6) which gave a satisfactory linear plot. This suggests that one essential arginine residue per monomer is involved in the catalytic function of ATP citrate lyase. A value of 0.68 was obtained for $\alpha = (k_1/k_2)$ from the slope $(\alpha - 1/i)$ of the curve, which suggests that the rate of modification of the essential arginine residue is about 1.5-fold faster than for other modifiable arginyl residues. Fig. 6, inset, shows the relation between the enzyme activity remaining, a , and the number of arginine residues modified, m , that was calculated from eqn. (1) with $n = 8.5$, $p = i = 1$ and $\alpha = 0.68$. The experimental values obtained were also plotted. The experimental curve agreed closely with the calculated values.

Fig. 6. Analysis of ATP citrate lyase-phenylglyoxal reaction data by the method of Tsou (1962)

Experimental conditions are the same as in Fig. 5. The left-hand component in eqn. (2) was calculated assuming $n = 8.5$, $p = i = 1$ and plotted against $\log(a)$. From the slope of the curve the value of α can be determined. Inset, the relationship between number of arginine residues modified (m) and fraction of enzyme activity (a). Using eqn. (1) with $n = 8.5$, $p = i = 1$ and $\alpha = 0.68$ the number of arginine residues modified for each value of a were calculated and plotted (●) and for comparison the experimentally obtained values (Δ) were also plotted.

Discussion

This paper describes the effects of arginine-specific reagents, phenylglyoxal and butanedione, on

ATP citrate lyase. Phenylglyoxal caused a rapid inactivation of ATP citrate lyase, the reaction being pseudo-first-order. Butanedione inactivated enzyme activity but to a lesser degree, and cyclohexanedione was least effective. Butanedione has been shown by others (Riordan, 1973; Chollet, 1978; Cipollo & Dunlap, 1978; Jordan & Wu, 1978; McTigue & Vanetten, 1978; Rogers *et al.*, 1978) and in this report to have maximal effects in borate buffer, possibly due to stabilization of the reaction product of the butane-dione-guanidino group by esterification with borate (Riordan, 1973). Inactivation by phenylglyoxal was lowest in borate buffer, and the most rapid inactivation was observed in Hepes buffer, pH 8.0. Inactivation by the α -dicarbonyl reagents appeared to be a direct consequence of the modification of enzyme arginine residues.

Kinetic analysis of the phenylglyoxal-modified ATP citrate lyase showed that the K_m values for the substrates were not significantly altered. The V decreased with inactivation in a pseudo-first-order fashion. This suggests that modification of the enzyme by phenylglyoxal or butanedione completely abolishes enzyme activity and that any residual activity in treated samples is the activity derived from unmodified molecules with normal active sites.

There are approx. 40 arginine residues per mol of enzyme monomer (Srere, 1972). Binding data showed that approx. 17 mol of phenylglyoxal were bound per subunit. Our data is consistent with the notion that for every two mol of phenylglyoxal bound to protein, one mol of arginine residue is modified (Takahashi, 1968; Daemen & Riordan, 1974; Lobb *et al.*, 1975; Schloss *et al.*, 1978). Anionic substrates (Mg-ATP, citrate and CoASH) protected the enzyme against inactivation, though to differing degrees. At concentrations less than 0.5 mM, Mg-ATP reduced the inactivation by phenylglyoxal and butanedione but at concentrations higher than 0.5 mM, Mg-ATP increased the rate of inactivation. The ambiguity in protection by Mg-ATP cannot be explained by our data. Higher concentrations of Mg-ATP may bring conformational changes at the active site of the enzyme, thus rendering essential arginine more reactive towards α -carbonyl reagents. CoASH and CoASH plus citrate gave excellent protection from inactivation by phenylglyoxal and butanedione. The binding data showed that of the 8.5 arginine residues of the enzyme susceptible to phenylglyoxal modification, 2–3 residues were protected by substrates. The combination of substrate-induced conformational changes and direct binding of the anionic groups of the substrates to arginine residues may be responsible for this protection.

The number of essential amino acid residues

required for catalytic activity has been determined for pyridoxamine phosphate oxidase (Horiike *et al.*, 1979), pepsin (Paterson & Knowles, 1972) and transferrin (Rogers *et al.*, 1978) by analysing the data produced by chemical modification with specific amino acid modifiers by the statistical method of Tsou (1962). By a similar analysis, we determined that each ATP citrate lyase subunit has an essential arginine residue required for catalytic function. As discussed by Horiike *et al.* (1979), the method for the determination of the number of essential amino acid residues from a plot of a as a function of m by extrapolation of the initial linear portion of the curve to 100% inactivation, can be used only when $\alpha = 1$ and $n(=p) = i = 1$ or $\alpha \ll 1$ and $p = i = 1$. Since in the present case α was calculated to be 0.68, this extrapolation method was not used. The order of reaction obtained with phenylglyoxal and butanedione was 1, indicating that one arginine residue is modified at the active site to produce inactivation.

The reaction catalyzed by ATP citrate lyase involves a complex series of transformations at the active site, possibly involving phosphoryl-enzyme (covalent) enzyme-citryl phosphate (noncovalent), citryl-enzyme (covalent) and enzyme-citryl-CoA (noncovalent) intermediates. The last-named undergoes Claisen cleavage, giving rise to the products oxaloacetate and acetyl-CoA carbanion, which finally is converted to acetyl-CoA (Walsh & Spector, 1969; Walsh, 1979). The glutamate γ -carboxyl group was suggested as the nucleophile involved in the formation of both phosphoryl-enzyme and citryl-enzyme (Suzuki *et al.*, 1969; Suzuki, 1971). In contrast, the phosphate residue in the phospho-enzyme was identified as histidyl N -phosphate (Cottam & Srere, 1969; Ramakrishna & Benjamin, 1979a). From the studies on modification of arginine residues, enzyme inactivation cannot be explained directly in terms of the Walsh (1979) reaction mechanism. However, modification of arginine residues in the close vicinity of the active site might make the nucleophilic group less approachable to the electrophilic groups of the substrate and thus cause inhibition. Alternatively, arginine could be the base involved in Claisen cleavage of citryl-CoA that is modified by α -dicarbonyl reagents; hence leading to inhibition of enzyme activity. However, more experimental data is needed to support any of these assumptions.

The bulky nature of substitution (about 8 arginine residues modified per subunit) could bring about conformational changes in the enzyme structure which could at the most cause partial inhibition but not complete inhibition. In majority of the cases studied, enzyme inactivation by phenylglyoxal and butanedione was shown to be due to modification of an essential arginine residue which prevents binding

of the substrate to the enzyme (Berghauser, 1977; Riordan *et al.*, 1977; Jordan & Wu, 1978; Philips *et al.*, 1978; Rogers *et al.*, 1978; Vensel & Kantrowitz, 1980). It is not known whether ATP citrate lyase has the same or different binding sites for different substrates. Complete loss of enzyme activity by treatment with both phenylglyoxal and butanedione suggests that an arginine residue(s) which interacts with substrates at the binding site (possibly CoASH binding site) was modified. Additional evidence for this hypothesis was the finding that the modified enzyme failed to bind to a CoA-agarose column and the excellent protection afforded by CoASH. Protection was minimal with citrate, and Mg-ATP at higher concentrations failed to protect the enzyme. In view of the complexities of the protection patterns noted, we were not able to determine if arginine residue(s) are involved in the binding of ATP and citrate to ATP citrate lyase. However, our data strongly suggest that the inactivation of ATP citrate lyase by phenylglyoxal and butanedione is at least in part due to the modification of arginine residues at the CoASH binding site.

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