Inactivation of chicken liver pyruvate carboxylase by 1,10-phenanthroline

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Inactivation of chicken liver pyruvate carboxylase by the chelating agent 1,10-phenanthroline follows pseudo-first-order kinetics. The hyperbolic dependence of the apparent first-order rate constant on 1,10-phenanthroline concentration is consistent with a two-step inactivation mechanism, in which 1,10-phenanthroline binds firstly to the enzyme, and secondly to the enzyme-bound Mn(II) ion. Binding of 1,10-phenanthroline to pyruvate carboxylase results in complete loss of ATP/P_i exchange activity, but only a 61 % decrease in pyruvate/oxaloacetate exchange activity. The rate of inactivation is greater at low enzyme concentrations, implying that binding of 1,10-phenanthroline to monomers and dimers is preferred relative to that of tetramers. Furthermore, in the presence of acetyl-CoA, which stabilizes the tetrameric structure, no dependence of inactivation on enzyme concentration is observed. As monitored by gel-permeation liquid chromatography, formation of the enzyme–Mn(II)–phenanthroline complex results in loss of the tetrameric structure of the enzyme. From atomic-absorption measurements, inactivation by 1,10-phenanthroline also causes some loss of Mn(II) from the enzyme. It is concluded that the Mn(II) atom does not participate directly in the reaction mechanism, but may play a structural role essential to the integrity of the enzyme's tetrameric structure.

INTRODUCTION

Pyruvate carboxylase (EC 6.4.1.1) catalyses the ATP-dependent carboxylation of pyruvate to form oxaloacetate in two partial reactions (1 and 2) linked by enzyme (E)-bound *N*-carboxybiotin:

(Wood & Barden, 1977). Previous studies have implicated a dissociable Mg(II) ion in the first partial reaction, and an intrinsic Mn(II) ion in the second partial reaction. Thus Mg(II) and MgATP²⁻ combined with sheep kidney pyruvate carboxylase in random order, but the binding of one reactant facilitated the binding of the other, and both were required for carboxylation of biotin to occur (Bais & Keech, 1972). Similar kinetics have been observed with both rat and chicken liver enzymes (McClure *et al.*, 1971; Barden & Scrutton, 1974).

Although 20 years have elapsed since the demonstration of tightly bound Mn(II) in chicken liver pyruvate carboxylase (Scrutton *et al.*, 1966), the role of Mn(II) in the enzyme's reaction mechanism is still uncertain. Studies of the effect of the metal ion on the longitudinal relaxation rate of water protons in the presence and absence of substrates have suggested that the Mn(II) ion is associated with the second partial reaction site (Scrutton *et al.*, 1973). However, pyruvate does not

appear to be an inner-sphere Mn(II) ligand, since a minimum Mn(II)–pyruvate distance of 0.6–0.7 nm has been obtained by both ¹³C (Fung *et al.*, 1973) and ¹H (Reed & Scrutton, 1974) n.m.r. spectroscopy.

Definition of the role of both Mg(II) and Mn(II) ions in the two partial reactions would be facilitated by a

study of the interaction of other metal ions with apopyruvate carboxylase. Consequently, an investigation of the effect of various chelating agents on the enzyme was undertaken as a preliminary step towards formation of the apoenzyme. In the course of these investigations 1,10-phenanthroline was observed to inactivate the enzyme rapidly and irreversibly. The results of a detailed study of the mechanism of 1,10-phenanthroline inactivation and the effect it has on the structure of the enzyme are reported in the present paper.

MATERIALS AND METHODS

Chemicals

AnalaR-grade 1,10-phenanthroline and laboratory-reagent-grade 8-hydroxyquinoline-5-sulphonic acid and pyridine-2-carboxylic acid were from BDH, Poole, Dorset, U.K. 1,7- and 4,7-Phenanthroline were from ICN Pharmaceuticals, Plainview, NY, U.S.A. Pyridine-

Abbreviation used: DTE, dithioerythritol.

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2,6-dicarboxylic acid was from Fluka, Buchs, Switzerland. Mops, *N*-ethylmorpholine, Tris, avidin and dithioerythritol (DTE) were from Sigma Chemical Co., St. Louis, MO, U.S.A. Hepes and Bicine were from Calbiochem-Behring, La Jolla, CA, U.S.A. (+)-[carboxy-¹⁴C]Biotin was from The Radiochemical Centre, Amersham, Bucks., U.K.

Chicken liver pyruvate carboxylase was prepared as described by Goss *et al.* (1979), except that a column of DEAE-Sephadex (69 cm × 2.5 cm) replaced the DEAE-Sephadex column. Only enzyme of purity greater than 95% [as judged by spectrophotometric scanning of SDS/polyacrylamide gels (Laemmli, 1970) after staining with Coomassie Brilliant Blue] and specific activity greater than 25 units/mg was used in this work.

Assays

Pyruvate carboxylase activity was assayed at pH 7.8 as described by Utter & Keech (1963). The ATP/P_i and pyruvate/oxaloacetate exchange reactions were assayed as described by Ashman & Keech (1975). One unit of activity is defined as the amount of enzyme catalysing the formation of 1 μmol of oxaloacetate/min at 25 °C. Biotin was assayed as described by Rylatt *et al.* (1977).

Liquid chromatography

Gel-permeation liquid chromatography was undertaken in a Pharmacia LCC500 FPLC system with a u.v.-absorption detector and a Superose 12 column operating at a flow rate of 0.5 ml/min. The elution buffer was 0.1 M-Tris/HCl/0.1 M-KCl/5 mM-DTE, pH 7.0. The calibration of the column for molecular size versus elution volume was determined by using standards covering a wide range of molecular size (thyroglobulin, 650 kDa, 7.8 ml; bovine serum albumin dimer, 132 kDa, 10.2 ml; bovine serum albumin monomer, 66 kDa, 11.6 ml; chymotrypsinogen, 24 kDa, 14.2 ml).

Atomic absorption spectroscopy

Analysis of the manganese content of the fractions eluted from the column was undertaken by atomic absorption spectroscopy using a Perkin-Elmer 430 atomic absorption spectrophotometer incorporating an HGA 400 graphite furnace. The spectrophotometer was calibrated with BDH MnCl₂ stock solutions diluted into the elution buffer used for liquid chromatography.

RESULTS AND DISCUSSION

Effect of various chelating agents on pyruvate carboxylase overall activity

A number of common chelating agents irreversibly inactivate pyruvate carboxylase activity, the most effective of the compounds studied being 1,10-phenanthroline (Table 1). The inactivation by 1,10-phenanthroline proceeds to at least 99% and follows pseudo-first-order kinetics with a rate constant of $3.95(\pm 0.14) \times 10^{-2} \text{ min}^{-1}$ at 25 °C, pH 7.0, at a phenanthroline concentration of 10 mM, and an enzyme concentration of 9.0 units/ml. The non-chelating analogues 1,7- and 4,7-phenanthroline (Table 1) and the Zn(II)-(1,10-phenanthroline)₂ complex (results not shown) have significantly smaller effects on pyruvate carboxylase activity, suggesting that inactivation is principally related to metal-ion chelation.

Two distinct mechanisms for enzyme inactivation by 1,10-phenanthroline have been reported. For example,

Table 1. Effect of various chelating agents on overall pyruvate carboxylase activity

Samples of pyruvate carboxylase (9.0 units/ml) were incubated at 25 °C in 50 mM-Mops/NaOH, pH 7.0, containing 5 mM-DTE, 32 mM-sucrose (present owing to dilution from enzyme storage buffer) and various chelating agents at the concentrations indicated. Samples were assayed at various time intervals for residual activity as described in the Materials and methods section. Rate constants (*k*) were obtained by a least-squares fit of the data to the equation for first-order inactivation, and are means ± S.E.M. Sucrose decreases the rate of inactivation by 10 mM-1,10-phenanthroline: e.g. for enzyme at 10.9 units/ml in 50 mM-Tris/HCl/5 mM-DTE, pH 7.0, $k = 4.98 (\pm 1.28) \times 10^{-2}$ and $1.90 (\pm 0.11) \times 10^{-2} \text{ min}^{-1}$ respectively in the absence and the presence of 160 mM-sucrose.

Chelating agent	Concn. (mM)	$10^3 \times k$ (min ⁻¹)
Buffer alone	—	0.29 ± 0.17
1,10-Phenanthroline	10	39.5 ± 1.4
1,7-Phenanthroline	2.7 (saturated)	1.76 ± 0.66
4,7-Phenanthroline	10	1.12 ± 0.17
8-Hydroxyquinoline-5-sulphonic acid	10	5.76 ± 0.72
Pyridine-2-carboxylic acid	100	5.94 ± 0.69
Pyridine-2,6-dicarboxylic acid	100	6.11 ± 0.53
EDTA	100	1.15 ± 0.25

inactivation of rabbit muscle aldolase is caused by oxidation of thiol groups to disulphides by atmospheric oxygen, which is catalysed by a Cu(II)-1,10-phenanthroline complex (Kobashi & Horecker, 1967; Kobashi, 1968). No inactivation of aldolase occurred in the presence of EDTA, and inactive enzyme was fully reactivated by treatment with mercaptoethanol or DTE. In contrast, the instantaneous inactivation of horse liver alcohol dehydrogenase by 1,10-phenanthroline (Vallee & Coombs, 1959) has been shown by X-ray crystallography to be due to formation of a complex between the active-site Zn(II) ion and the chelating agent (Boiure & Branden, 1977).

Since chicken liver pyruvate carboxylase (i) is inactivated by thiol reagents (Palacian & Neet, 1970), and (ii) contains a tightly bound Mn(II) ion (Scrutton *et al.*, 1966), as well as requiring a bivalent cation, other than that bound to ATP, in the overall reaction (Barden & Scrutton, 1974), either mechanism appeared possible. However, since inactivation of pyruvate carboxylase by 1,10-phenanthroline is observed in the presence of DTE and EDTA (see below), the oxidative mechanism may be eliminated, suggesting that inactivation arises from metal chelation.

Treatment of pyruvate carboxylase with 10 mM-EDTA at pH 7.2 removes all metal ions other than the tightly bound Mn(II) ion (Scrutton *et al.*, 1966), but does not affect the rate of inactivation subsequently induced by 1,10-phenanthroline. Thus the first-order rate constant (*k*) for the inactivation of pyruvate carboxylase that had previously been treated with 10 mM-EDTA at 25 °C, pH 7.2, for 24 h is $2.04(\pm 0.04) \times 10^{-2} \text{ min}^{-1}$ at an enzyme concentration of 13.4 units/ml. Under the same condi-

tions the untreated sample has a k value of $2.39(\pm 0.17) \times 10^{-2} \text{ min}^{-1}$. Similarly, the presence of 10 mM-EDTA in the inactivation mixture does not affect the rate of inactivation [$k = 2.16(\pm 0.08) \times 10^{-2} \text{ min}^{-1}$]. It therefore appears probable that the tightly bound Mn(II) ion, rather than the metal ion involved in the first partial reaction, is the site for 1,10-phenanthroline chelation. In agreement with this, inactivation by 1,10-phenanthroline leads to a loss of Mn(II) (see below). Likewise, in pyruvate carboxylase from bakers' yeast, which contains a bound Zn(II) ion instead of Mn(II), inactivation with the metal chelator 8-hydroxyquinoline-5-sulphonate results in a concomitant loss of Zn(II) (Scrutton *et al.*, 1970). Free Mn(II) and Zn(II) ions both form very stable 1:1 complexes with 1,10-phenanthroline, with stability constants of $3.2 \times 10^4 \text{ M}^{-1}$ and $2.0 \times 10^6 \text{ M}^{-1}$ respectively (Irving & Mellor, 1962). A direct test of the hypothesis that enzyme inactivation is due to Mn(II) ion chelation, by spectral studies similar to those used with Zn(II)-containing enzymes (Vallee *et al.*, 1958; Drum & Vallee, 1970), is not feasible because the binding constant for 1,10-phenanthroline chelation to the enzyme-bound Mn(II) is very small (see below), and therefore observation of any changes in the u.v. spectrum of bound phenanthroline is not possible at the concentrations required for inactivation.

Effect of 1,10-phenanthroline concentration on inactivation rate

Extensive studies of the inactivation of horse liver alcohol dehydrogenase by 1,10-phenanthroline have shown that the chelating agent (L) first binds to the enzyme (EM) to form a binary complex (LEM) which does not involve inner-sphere chelation of the active-site Zn(II) ion (Frolich *et al.*, 1978). Dissociation of an inner-sphere H_2O ligand is then the rate-limiting step in formation of an inner-sphere 1,10-phenanthroline-Zn(II)-enzyme complex (EML) (Frolich *et al.*, 1978; Evans & Shore, 1980) as shown in Scheme 1:

Direct Zn(II)-1,10-phenanthroline co-ordination has been observed by X-ray crystallography (Boiure & Branden, 1977). Similar complexes are formed between Co(II)-bovine carbonic anhydrase and 5-methyl-1,10-phenanthroline (Kidani & Hirose, 1978) and Zn(II)-insulin or Zn(II)-7 S nerve growth factor and terpyridine (Dunn *et al.*, 1980).

If it is assumed that both EM and LEM are active, but that EML is inactive (i.e. that the conversion of EML into LEM is not feasible), then it can be shown that the rate equation predicted by the mechanism of Scheme 1 is:

$$-\frac{dA}{dt} = \frac{k_2 L}{K_L + L} A$$

where $-dA/dt$ is the rate of loss of activity, A is the concentration of the active enzyme species EM and LEM, k_2 is the rate constant for formation of the EML complex and K_L is the dissociation constant of the LEM complex. Thus the rate of loss of activity is first order, and the apparent first-order rate constant, $k_{\text{app.}} [= k_2 L / (K_L + L)]$, will exhibit a hyperbolic dependence on the concentration of L, so that a plot of $k_{\text{app.}}^{-1}$ versus L^{-1} will be a straight line with slope K_L/k_2 and intercept k_2^{-1} .

Fig. 1. Effect of 1,10-phenanthroline concentration on inactivation rate in the presence of various substrates and products

The inactivation rate of pyruvate carboxylase (8.9 units/ml) at 25 °C was determined in the presence of various concentrations (0.99–10.27 mM) of 1,10-phenanthroline, 32 mM-sucrose and the compounds indicated. At various times, samples were assayed for residual activity as described in the Materials and methods section. From a plot of percentage activity versus time, the rate of inactivation, $k_{\text{app.}}$, was determined from a least-squares fit of the experimental points to the equation for first-order inactivation. In the presence of a particular compound, the Figure shows a plot of the dependence of $k_{\text{app.}}^{-1}$ on $[1,10\text{-phenanthroline}]^{-1}$. From these plots the values of K_L and k_2 could be determined (see the text). □, Control (buffer alone); ●, plus 10 mM- NaHCO_3 ; △, plus 10 mM- Na_2HPO_4 ; ▽, plus 10 mM-sodium pyruvate; ○, plus 0.5 mM- Na_2ADP ; ■, plus 0.5 mM- Na_2ATP ; ▼, plus 0.05 mM-acetyl-CoA.

The inactivation of pyruvate carboxylase by 1,10-phenanthroline follows this rate equation closely, suggesting a two-step inactivation mechanism. Loss of activity is pseudo-first-order, and a double-reciprocal plot of $k_{\text{app.}}^{-1}$ versus L^{-1} is a straight line (Fig. 1). The values of K_L and k_2 obtained for pyruvate carboxylase in the absence of any ligand (7.04 mM and $1.81 \times 10^{-2} \text{ min}^{-1}$ respectively) are both considerably different from the values found for alcohol dehydrogenase (12 μM and $1.0 \times 10^4 \text{ min}^{-1}$ respectively; Frolich *et al.*, 1978). The value of k_2 for alcohol dehydrogenase is itself two orders of magnitude lower than the rate of water dissociation from simple zinc complexes (Dunn, 1976). N.m.r. relaxation data suggest that no water molecules are sufficiently close to the Mn(II) ion to be considered inner-sphere ligands (Scrutton *et al.*, 1973). A minimum Mn-H distance of 0.35 nm, consistent with outer-sphere co-ordination, was calculated on the basis of one water

molecule acting as a Mn(II) ligand. Dissociation of this water molecule would be expected to be rapid, and therefore the small value observed for k_2 suggests that for pyruvate carboxylase the rate of inactivation may be limited by the rate of dissociation of one of the amino acid side chains which acts as a Mn(II) ligand, or possibly by an even more indirect means.

Fig. 1 also shows reciprocal plots for inactivation in the presence of various substrates or products, the concentrations of which were chosen to give reasonable inactivation rates. Except for sodium pyruvate, all plots had similar intercepts on the ordinate. Thus, within experimental error, the values of k_2 obtained in the presence of Na_2HPO_4 , NaHCO_3 , Na_2ADP , Na_2ATP and acetyl-CoA and in their absence were the same, and so the binding of the 1,10-phenanthroline ligand to the Mn(II) ion is not affected by the presence of these species. However, since the values of K_L vary for each species, the binding of 1,10-phenanthroline to the enzyme is altered by their presence, suggesting that the different conformational effects associated with binding of a particular substrate, product or acetyl-CoA to the enzyme have a variable effect on the binding of 1,10-phenanthroline.

Fig. 1 suggests that binding of 1,10-phenanthroline to the Mn(II) ion is affected by the presence of pyruvate. Pyruvate is the only one of the substrates/products tested that is involved in the second partial reaction, and its variable behaviour may be due to the different effects that 1,10-phenanthroline has on both partial reactions (see below).

Effects of 1,10-phenanthroline on the two partial reactions

1,10-Phenanthroline has a differential effect on the two partial reactions (see eqns. 1 and 2) catalysed by pyruvate

carboxylase. Inactivation of the first partial reaction, measured by the ATP/ P_i exchange reaction, follows pseudo-first-order kinetics with a half-life identical with that measured simultaneously for the overall reaction (36 min at an enzyme concentration of 23.5 units/ml; Fig. 2a). Inactivation of the second partial reaction, measured by the pyruvate/oxaloacetate exchange reaction, does not follow pseudo-first-order kinetics (Fig. 2b). If it is assumed that the pyruvate carboxylase-Mn(II)-1,10-phenanthroline complex retains fractional activity f in the pyruvate/oxaloacetate exchange reaction, then the percentage residual exchange activity at time t is given by the expression $100[(1-f)e^{-kt} + f]$, where k is the first-order rate constant for inactivation of the overall reaction. The line in Fig. 2(b) which represents the best fit of the experimental points to this equation, was obtained with $f = 0.39$. It thus appears that formation of the pyruvate carboxylase-Mn(II)-1,10-phenanthroline complex results in complete loss of ATP/ P_i -exchange activity, but only a 61% decrease in the pyruvate/oxaloacetate exchange activity. The most obvious corollary of this observation is that the Mn(II) atom of pyruvate carboxylase does not participate directly in the second partial reaction. This conclusion is consistent with the interpretation of previous n.m.r. experiments on pyruvate carboxylase (Scrutton *et al.*, 1973; Fung *et al.*, 1973; Reed & Scrutton, 1974). When bound to the enzyme, the substrate pyruvate is too far away from the Mn(II) ion to be an inner-sphere ligand. Moreover, since no water molecules are present in the inner co-ordination sphere of the metal ion (Scrutton *et al.*, 1973), the intermediate participation of a tightly bound water molecule in the abstraction of a proton from pyruvate by the Mn(II) ion appears unlikely. Further evidence for the absence of a functional role for the Mn(II) ion in the mechanism of the second partial reaction is provided by the observation

Fig. 2. Effect of 1,10-phenanthroline on the partial reactions of pyruvate carboxylase

Samples of pyruvate carboxylase (23.5 units/ml) were incubated at 25 °C in 50 mM-Mops/NaOH, pH 7.0, containing 5 mM-DTE, 80 mM-sucrose and 10 mM-1,10-phenanthroline. At the times indicated, samples were assayed for residual overall activity (●) or for residual activity in the ATP/ P_i (▲) (a) and pyruvate/oxaloacetate (■) (b) partial reactions as described in the Materials and methods section. The lines indicate the least-squares fit of the experimental points for residual activity to the equation for first-order inactivation, except for the pyruvate/oxaloacetate exchange reaction. In this case the line represents the best fit of the experimental points to the equation $y = (1-f)e^{-kt} + f$, where k is the first-order rate constant for inactivation of the overall reaction. The best-fit value of f , the fractional residual exchange activity, was 0.39.

that pyruvate carboxylase substituted with Mg(II) instead of Mn(II) has only a moderately increased K_m for pyruvate and K_i for the inhibitor, oxalate (Scrutton *et al.*, 1972).

The Mn(II) ion is also unlikely to be involved directly in the first partial reaction since, from e.p.r. studies, the binding site for Cr(III)ATP⁻ is distant (~1 nm) from the Mn(II) ion (Reed & Scrutton, 1974). The inactivation of the first partial reaction upon 1,10-phenanthroline binding is most likely therefore to be due to structural changes associated with the ligand binding to the enzyme (see below).

Effects of pyruvate carboxylase concentration, acetyl-CoA and other ligands on inactivation rate

As discussed above, the inactivation of pyruvate carboxylase by 1,10-phenanthroline is first-order with respect to enzyme. The rate constant derived from such first-order plots is normally independent of enzyme concentration, but for pyruvate carboxylase in the absence of acetyl-CoA the apparent first-order rate constant increased with decreasing enzyme concentration (Table 2). Although it would have been desirable to obtain data at lower enzyme concentrations, the chicken enzyme, like the sheep enzyme (Ashman *et al.*, 1972), undergoes irreversible dilution-inactivation at low enzyme concentrations. Dilution-inactivation, like cold-inactivation (Irias *et al.*, 1969), involves dissociation of the tetrameric enzyme into dimers and monomers (Khew *et al.*, 1983), and thus the observed dependence of the apparent first-order rate constant on enzyme concentration is consistent with more rapid inactivation being due to preferred binding of 1,10-phenanthroline to the dimer and monomer species. This explanation is supported by the observation that in the presence of acetyl-CoA, which prevents both cold- and dilution-inactivation by stabilizing the tetramer (Irias *et al.*, 1969; Khew *et al.*, 1983), the apparent first-order rate constant is independent of enzyme concentration (Table 2). Furthermore, even at high enzyme concentrations, this rate constant is less than that in the absence of acetyl-CoA, implying that the subtle alterations in the tetrameric

structure caused by acetyl-CoA (Mayer *et al.*, 1980; Attwood *et al.*, 1986) makes the Mn(II) ion less accessible to 1,10-phenanthroline.

Table 3 and Fig. 1 show that, for a fixed substrate or product concentration, the rate of inactivation of pyruvate carboxylase by 1,10-phenanthroline is decreased compared with that in the absence of a ligand. The effectiveness of the compounds tested in preventing inactivation by 1,10-phenanthroline (Table 3, Fig. 1) is paralleled by their ability to prevent cold-inactivation, with the sole exception of pyruvate, which slightly increases the rate of cold-inactivation (Irias *et al.*, 1969). Furthermore, 1.5 M-sucrose and 2 M-KCl, which both provide complete protection against cold-inactivation, also prevent inactivation by 1,10-phenanthroline (results not shown).

The greatest degree of protection against inactivation was afforded by ATP and ADP (Table 3), which bind at the first partial reaction site. Furthermore, phosphate, a product of the first partial reaction, also protects markedly against inactivation. Thus, when 10 mM-1,10-phenanthroline was added to pyruvate carboxylase at 8 units/ml in 50 mM-sodium phosphate or potassium phosphate buffer (pH 7.0)/5 mM-DTE/32 mM-sucrose, the rate of inactivation was identical with that of a solution containing no 1,10-phenanthroline (results not shown). Fig. 3 demonstrates that increasing concentrations of sodium phosphate in 50 mM-Mops, pH 7.0, decrease the rate of inactivation, so that at 30 mM-sodium phosphate the enzyme is almost completely protected. It is concluded that the lack of inactivation in phosphate buffer is a function purely of that buffer system, since in 50 mM solutions of Hepes, Tris, *N*-ethylmorpholine, Mops and Bicine, pH 7.0, inactivation by 1,10-phenanthroline occurred at a comparable rate (results not shown).

Effect on the structure of pyruvate carboxylase owing to inactivation by 1,10-phenanthroline

Evidence suggesting that inactivation of pyruvate carboxylase by 1,10-phenanthroline is associated with loss of the tetrameric structure of the enzyme and of

Table 2. Effect of pyruvate carboxylase concentration on inactivation rate with and without acetyl-CoA

The first-order rate constants (k) for the inactivation of pyruvate carboxylase by 1,10-phenanthroline at various concentrations of enzyme in the absence and presence of 0.25 mM-acetyl-CoA were determined as described in the legend to Fig. 1. Results are means $\pm$ S.E.M.

[Pyruvate carboxylase] (units/ml)	$10^3 \times k$ (min ⁻¹)	
	With acetyl-CoA	Without acetyl-CoA
0.68	5.1 $\pm$ 0.3	
1.37	6.3 $\pm$ 0.2	
5.69	5.3 $\pm$ 0.3	
13.4		20.4 $\pm$ 0.4
24.1		11.0 $\pm$ 0.9
26.7	5.5 $\pm$ 0.5	
56.1		8.3 $\pm$ 0.6

Table 3. Effect of substrates and products on the inactivation of pyruvate carboxylase activity by 1,10-phenanthroline

Samples of pyruvate carboxylase (9.0 units/ml) were incubated at 25 °C in 50 mM-Mops/NaOH, pH 7.0, containing 5 mM-DTE, 32 mM-sucrose, 10 mM-1,10-phenanthroline and test compound at a concentration of 10 mM, except for acetyl-CoA (0.25 mM). Samples were assayed at various time intervals for residual activity as described in the Materials and methods section. Rate constants (k) were obtained by a least-squares fit of the data to the equation for first-order inactivation, and are means $\pm$ S.E.M.

Compound	$10^3 \times k$ (min ⁻¹)
—	39.5 $\pm$ 1.4
NaHCO ₃	39.8 $\pm$ 1.6
oxaloacetate (disodium salt)	12.1 $\pm$ 2.1
Pyruvate (sodium salt)	11.8 $\pm$ 1.0
Na ₂ HPO ₄	24.5 $\pm$ 0.3
Na ₂ ATP	4.3 $\pm$ 0.2
Na ₂ ADP	4.4 $\pm$ 0.2
Acetyl-CoA	5.3 $\pm$ 0.2

Fig. 3. Inactivation of pyruvate carboxylase by 1,10-phenanthroline in the presence of various concentrations of phosphate

Samples of pyruvate carboxylase (7.5 units/ml) were incubated at 30 °C in 50 mM-Mops/NaOH, pH 7.0, containing 5 mM-DTE, 32 mM-sucrose and a concentration of (▲) 0, (●) 10 mM-, (■) 30 mM- and (◇) 50 mM-sodium phosphate, pH 7.0. At the times indicated, samples were assayed for residual activity and the rate of inactivation was determined from the least-squares fit of the experimental points to the equation for first-order inactivation.

Mn(II) is indicated by the chromatographic profiles after inactivation and from determination of the Mn(II) and biotin contents of the various protein species present (Fig. 4). When pure tetrameric enzyme at 241 units/ml was inactivated in the absence of acetyl-CoA by 1,10-phenanthroline to 33% of its original activity, some precipitation of enzyme occurred. Chromatography of the supernatant gave a profile (Fig. 4a) of species that corresponded to highly aggregated enzyme (peak 1), as well as peak 2 at an elution volume characteristic of tetrameric enzyme and peak 3 at a much higher volume corresponding to the elution of 1,10-phenanthroline. The letters T, D and M refer to the positions for elution of tetrameric, dimeric and monomeric enzyme. In the elution profiles of totally inactivated enzyme samples (results not shown) the dimeric peak was clearly resolved, since in this case little of the tetramer species was present.

Fig. 4. Liquid chromatography and atomic-absorption-spectrophotometric measurements on pyruvate carboxylase inactivated with 1,10-phenanthroline

The conditions used are outlined in the Materials and methods section. Enzyme (0.4 ml) at 413 units/ml was applied to the column, and the peak corresponding to tetrameric enzyme was collected. This was concentrated to 241 units/ml and inactivated with 10 mM-1,10-phenanthroline. As measured spectrophotometrically, after 2.5 h the enzyme had 33% of its original activity, and some precipitation of the enzyme had occurred. The supernatant (0.4 ml) was applied to the column and gave the profile shown in (a); 0.5 ml fractions were collected. Also shown in (a) are the concentrations of Mn(II) in each fraction as determined by atomic absorption spectrophotometry. The elution positions corresponding to tetrameric (T), dimeric (D) and monomeric (M) pyruvate carboxylase as determined from the calibration plot are indicated in (a). Peaks 1 and 2 represent aggregated and tetrameric enzyme, and peak 3 corresponds to that for 1,10-phenanthroline. In (b) the [Mn(II)]/[biotin] molar ratios for peaks 1 and 2 are presented.

The Mn(II) and biotin content of the three peaks in Fig. 4(a) was determined by atomic absorption spectrophotometry and by biotin assay (Rylatt *et al.*, 1977) respectively. Fig. 4(a) shows that some Mn(II) was eluted with the 1,10-phenanthroline (peak 3). The amount of Mn(II) recovered in all the fractions was only 19% of that loaded. As measured by the biotin content of the various fractions, the corresponding percentage of the protein recovered was 31%. It is therefore concluded that a significant quantity of Mn(II) remained on the column, and that inactivation of pyruvate carboxylase by 1,10-phenanthroline involves Mn(II) chelation and, to a limited extent, its subsequent removal from the enzyme.

The ratio of Mn(II) to biotin concentration is plotted in Fig. 4(b). The ratios for the fractions corresponding to the aggregated enzyme are much less than the expected value of around 1.0 observed for tetrameric enzyme. The fractions corresponding to the position for elution of dimeric and monomeric enzyme have Mn(II)/biotin ratios much lower than 1.0. Furthermore, the Mn(II)/biotin ratio for the precipitated enzyme was 0.66, which is a similar value to that for the aggregated-enzyme peak in Fig. 4(a). These data are indicative of a mechanism of inactivation by 1,10-phenanthroline involving dissociation of the tetrameric enzyme structure into dimers and monomers, with a concomitant loss of Mn(II), followed by coalescence into aggregated species. In their study of cold-inactivation of pyruvate carboxylase, Irias *et al.* (1969) described a similar mechanism which involved dissociation of the enzyme into monomeric species and subsequently aggregation. The very similar ability of the various substrates and products to protect against inactivation by cold and 1,10-phenanthroline (Table 3, Fig. 1) is consistent with this postulate. Likewise, at low enzyme concentrations the protection against inactivation afforded by acetyl-CoA, which protects against dissociation of tetramers (Table 2), is also consistent with the suggestion that dimers and monomers interact more readily with 1,10-phenanthroline.

The 1,10-phenanthroline-induced loss of tetrameric structure reported here for chicken liver pyruvate carboxylase is reminiscent of the dissociation of tetrameric molecules to dimers and monomers induced by exposure to EDTA of pyruvate carboxylase from *Rhizopus arrhizus* (Mayer *et al.*, 1985). Likewise, in transcarboxylase (EC 2.1.3.1), inactivation with 1,10-phenanthroline is accompanied by dissociation of the enzyme into inactive subunits (Ahmad *et al.*, 1972).

Thus, in conclusion, the inactivation of pyruvate carboxylase by 1,10-phenanthroline causes major changes to the quaternary structure of the enzyme. Since the Mn(II) ion is not directly involved in either partial reaction, the structural changes upon 1,10-phenanthroline binding lead to differential effects on both partial reactions (Fig. 2), implying that the Mn(II) ion has a structural rather than a catalytic role in pyruvate carboxylase.

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