EFFECT OF DICARBOXYLIC ACIDS ON INACTIVATION OF CHICKEN LIVER PYRUVATE CARBOXYLASE CAUSED BY INCUBATION AT 2°C

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Received 11 April 1974

1. Introduction

Previous studies have shown that various analogs of pyruvate and oxaloacetate act as specific inhibitors of the transcarboxylation partial reaction catalyzed by chicken liver pyruvate carboxylase and, as indicated by the reduction in $1/T_{1p}$* of water protons on formation of the enzyme–inhibitor complexes. bind to this pyruvate carboxylase in the vicinity of the bound Mn (II) [1,2]. Such binding is accompanied by a marked decrease in the Mn (II)–water proton dipolar interaction [1]. These inhibitors have been tentatively classified as Types I, II or III on the basis of differences in (i) the patterns of inhibition observed in initial rate studies when pyruvate was the varied substrate and (ii) the relationship between $1/T_{1p}$ and ligand concentration [1]. The studies described here provide some further basis for this classification of the inhibitors since the rate of inactivation observed on incubation of chicken liver pyruvate carboxylase at 2°C is decreased in the presence of Type I inhibitors, e.g. oxalate, fluorpyruvate, but is increased on addition of Type II and III inhibitors, e.g. malonate, L-malate. These data suggest that the differences in the inhibition patterns described previously [1] may be related to differing effects of these inhibitors on the conformation of pyruvate carboxylase.

* $1/T_{1p}$ is defined as the paramagnetic contribution to the longitudinal nuclear magnetic relaxation rate ($1/T_1$).

2. Materials and methods

Pyruvate carboxylase was purified from chicken liver mitochondria as described previously [3] and was assayed by measurement of oxaloacetate production in the presence of malate dehydrogenase and NADH [4].

The effect of inhibitors on the inactivation of pyruvate carboxylase caused by incubation at 2°C was examined essentially as described by Scrutton and Fung [3]. Purified pyruvate carboxylase (specific activity = 10–12 units/mg) was equilibrated with 0.001 M $(CH_3)_4N^+$-HEPES, pH 7.2 containing 0.6 M $(CH_3)_4NCl$ and 0.1 mM DTE by gel filtration on Sephadex G-25 (20 × 1 cm). After gel filtration the enzyme was diluted to a final concentration of 0.2–0.4 mg/ml in the same buffer containing the appropriate concentration of the inhibitor and held at 2°C or 25°C. Small aliquots (2–10 μl) of this incubation system were removed for assay of residual enzymic activity at various times over the period required for loss of at least 60% of the initial activity in the sample held at 2°C. Under the conditions employed in this study the rate of inactivation which is observed on incubation at 2°C was first order in enzyme sites for at least one half-life in the presence or absence of the various added components. Dissociation constants (K_d) and relative rates of inactivation (k_2/k_1) for the various complexes were obtained by analysis of the inactivation rate data according to eq. 1 [5]:

$$\frac{V_A}{V_O} = \frac{k_2}{k_1} + \frac{(1 - \frac{V_A}{V_O})}{(A)} \qquad (1)$$

In eq. 4 V_A and V_O are the first order rate constants describing the inactivation process in the presence and absence of a given concentration of the ligand, A, and k_2 and k_1 are the first order rate constants describing the rate of inactivation of the enzyme–ligand complex and the free enzyme respectively.

3. Results and discussion

Previous study by Irias et al. [6] have shown that pyruvate and oxaloacetate, the substrates of the transcarboxylation partial reaction catalyzed by pyruvate carboxylase, have qualitatively different effects on the rate of inactivation which is observed on incubation of chicken liver pyruvate carboxylase at 2°C. The data illustrated in fig. 1 indicate a similar qualitative difference in respect to the effect of Type I, and Types II and III, inhibitors of this partial reaction on this inactivation rate. Addition of a saturating concentration of oxalate (Type I) markedly depresses the rate of inactivation whereas addition of malonate (Type II) or L-malate (Type III) enhances this rate. From fig. 1 it is apparent that the rate of inactivation is first-order in enzyme sites in the presence, as well as the absence, of these ligands hence validating the procedure employed for subsequent analysis of the inactivation rate data according to eq. 1 [5].

When the rate of inactivation is measured as a function of ligand concentration for examples of all three classes of the inhibitors, and for oxaloacetate, a hyperbolic relationship is observed between inactivation rate and ligand concentration. This is illustrated in fig. 2 for oxalate and L-malate when the data are plotted according to eq. 1. In the plot shown a linear relationship is observed for both these ligands permit-

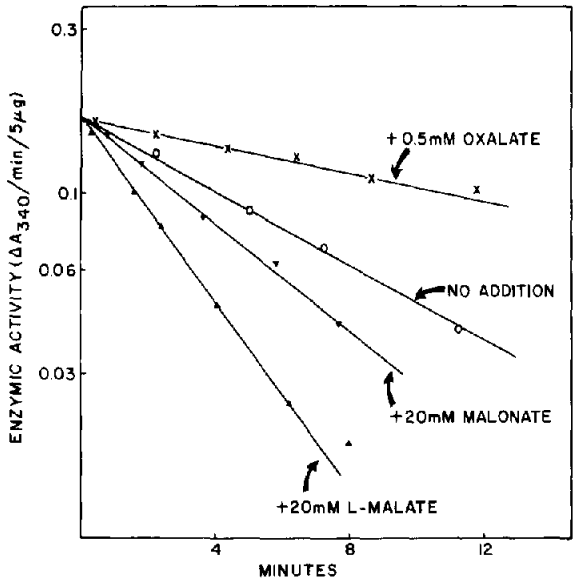

Fig. 1. Effect of various inhibitors on the rate of inactivation observed on incubation of chicken liver pyruvate carboxylase at 2°C. The incubation system contained 1 mM $(CH_3)_4{}^+N$ Hepes pH 7.2, 0.6 M $(CH_3)_4NCl$, 0.21 mg pyruvate carboxylase (specific activity = 11.4 units/mg) and the concentration of ligands as indicated in a total volume of 0.2 ml. The inactivation was initiated by addition of the enzyme to a pre-cooled system at 2°C and the extent of residual activity estimated at the times indicated by removal of an aliquot (2–5 μl) of this incubation system and dilution into 1.0 ml of the standard assay system [4]. Residual enzymic activity was estimated from the initial rate of decrease in A_{340}. Previous studies have shown that reactivation of pyruvate carboxylase does not occur under these conditions [6].

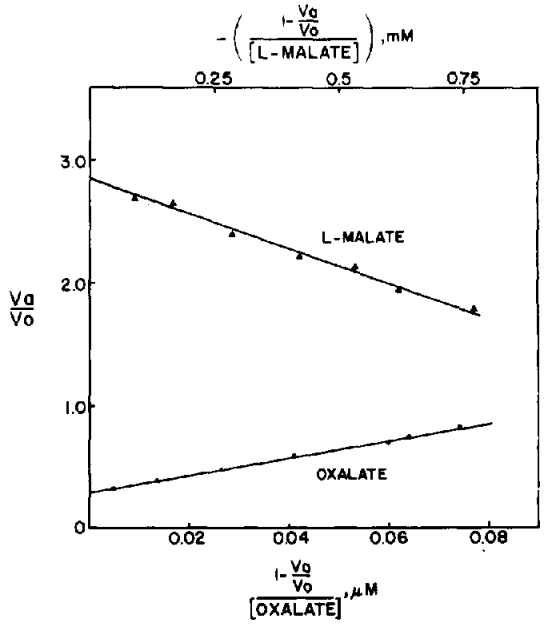

Fig. 2. Relationship between the rate of inactivation at 2°C and ligand concentration for oxalate and L-malate. Inactivation rates were estimated as a function of the concentrations of oxalate and L-malate using a protocol similar to that described for fig. 1. These data are plotted according to eq. 1 in the figure to give K_d for the enzyme–ligand complex (slope) and k_2/k_1, the rate of inactivation of the enzyme–ligand complex relative to that observed for the free enzyme (intercept) [15].

Table 1
Comparison of dissociation constants obtained from cold inactivation studies with dissociation constants obtained from measurement of $1/T_{1p}$ of solvent protons and with inhibition constants derived from initial rate studies

Ligand	Cold inactivation		$\triangle 1/T_{1p}$[a]	Inhibition[a]
	K_d (mM)	k_2/k_1	K_d (mM)	K_i (mM)
Inhibitors				
Type I				
Oxalate	0.007	0.3	0.003	0.011
Fluoropyruvate	0.21	0.4	0.23	0.17
Oxamate	1.21	0.3	1.45	1.6
Type II				
Malonate	3.5	1.5	6.7	5.4
Type III				
L-Malate	1.7	2.5	3.0	6.5
Substrates[b,c]				
Oxaloacetate	1.2	0	1.7	–

[a] From [1] and unpublished data.
[b] Although addition of 10 mM pyruvate causes a slight enhancement of the rate of inactivation at 2°C (cf. [6]) the magnitude of this effect is insufficient to permit determination of K_d and k_2/k_1 for this substrate.
[c] Assay for oxaloacetate after completion of the inactivation rate determination indicated that only a minimal decrease (< 10%) in concentration was observed over the time required for 60% decrease in enzymic activity in the sample incubated at 2°C.

ting estimation of K_d from the slope and k_2/k_1 from the ordinate intercept. Table I summarizes the values of K_d and k_2/k_1 obtained for oxaloacetate and for various Type I, II and III inhibitors. For purposes of comparison inhibitor and dissociation constants obtained for these ligands from initial rate and solvent proton relaxation rate studies respectively are also included in table 1. Reasonable agreement between the various constants is observed for each ligand suggesting that the effects observed on the rate of inactivation at 2°C result from formation of the same complex as that which participates in catalysis and which causes a decrease in the interaction between the bound Mn(II) and the solvent. Since previous studies have shown that inactivation caused by incubation at 2°C is accompanied by dissociation of this pyruvate carboxylase to inactive monomers, we may conclude that Type I inhibitors, e.g. oxalate, decrease the rate of inactivation by stabilizing the active tetramer whereas Type II and III inhibitors, e.g. malonate, L-malate, increase the inactivation rate as a result either of preferential interaction with an inactive tetramer or monomer, or of induction of a conformation which favors such dissociation. Furthermore comparison with the effect of the substrates of the transcarboxylation partial reaction on the rate of inactivation (table 1) [6] suggests that interaction of the enzyme which with Type I inhibitors may induce a conformation similar to that characteristic of the E–oxaloacetate complex. In contrast the complexes formed with Type II and III inhibitors may have a conformation which more closely resembles that of the E–pyruvate complex. This postulate is in accord with the previous proposal that oxalate acts as a transition state analog for pyruvate carboxylase [7]. It is of interest that the difference in conformation between Type I, and Type II and III inhibitor complexes detected by the cold inactivation studies (figs.

1 and 2) is also reflected in the EPR spectra of the bound Mn(II) obtained for these complexes. Addition of Type I inhibitors, e.g. oxalate, fluoropyruvate, has been found to cause marked narrowing of the individual resonance lines in the Mn(II)–EPR spectrum [2] whereas the width of these lines is unaffected or somewhat broadened in the presence of malonate or L-malate [8]. Thus the tightening of the enzyme conformation observed on addition of Type I inhibitors, which is indicated by the decreased rate of inactivation at 2°C (fig. 2), is also expressed as a decrease in the extent of interaction of solvent protons with the coordination sphere of the bound Mn(II) (cf. [2]).

Acknowledgements

I am grateful to Ms. Dawn White for valuable technical assistance and to Dr. George Reed for the EPR studies.

Supported in part by USPHS grant No. AM 15468 and NSF grant No. GB 30223.

References

[1] Mildvan, A.S., Scrutton, M.C. and Utter, M.F. (1966) J. Biol. Chem. 241, 3488–3498.
[2] Reed, G.H. and Scrutton, M.C. (1974) J. Biol. Chem. 249, in press.
[3] Scrutton, M.C. and Fung, C.H. (1972) Arch. Biochem. Biophys. 150, 636–647.
[4] Scrutton, M.C., Olmsted, M.R. and Utter, M.F. (1968) Methods in Enzymology 13, 235–249.
[5] Scrutton, M.C. and Utter, M.F. (1965) J. Biol. Chem. 240, 3714–3723.
[6] Irias, J.J., Olmsted, M.R. and Utter, M.F. (1969) Biochemistry 8, 5136–5148.
[7] Wishnick, M., Lane, M.D. and Scrutton, M.C. (1970) J. Biol. Chem. 245, 4939–4947.
[8] Reed, G.H. and Scrutton, M.C. Unpublished observations.