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PYRUVATE CARBOXYLASE: INACTIVATION BY SULFHYDRYL-GROUP REAGENTS IN THE PRESENCE OF CERTAIN INORGANIC ANIONS, SUBSTRATES, AND MODIFIERS AND THE EFFECTS ON THE ALLOSTERIC PROPERTIES OF THE ENZYME

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SUMMARY

Pyruvate carboxylase (pyruvate:CO_2 ligase (ADP), EC 6.4.1.1) from chicken liver reacts with sulfhydryl-group reagents, *N*-ethylmaleimide and L-cystine, in two phases. The first phase is accompanied by modification of 4 to 32 of the 55 sulfhydryl groups of the enzyme, depending on the reagent used and the inorganic anion present: phosphate, sulfate, or chloride. There is partial or no loss of activity and no gross change in the quaternary structure of the enzyme. During the second phase of modification, from 16 to 39 additional groups react, and complete inactivation takes place. This phase is accompanied by dissociation to monomers and/or formation of high molecular weight aggregates. The inorganic anion present determines the number of SH groups reacting with *N*-ethylmaleimide or cystine in each phase and the rate of the reaction. This diversity of reactivities is interpreted as being a consequence of the different conformations assumed by pyruvate carboxylase in the presence of either phosphate, sulfate, or chloride.

Two differently modified pyruvate carboxylases were obtained, after the first phase of reaction but under different conditions, by reaction of 32 or 16 cysteine residues with *N*-ethylmaleimide per molecule of enzyme. Both *N*-ethylmaleimide enzymes showed a decrease in the value of the Hill coefficient for acetyl-CoA from 2.7 (native enzyme) to 2.2. This lower value of the Hill coefficient indicates a decrease in the cooperativity of the activation by acetyl-CoA, and is interpreted as a decrease in the degree of interaction among the subunits of the enzyme. In contrast, pyruvate carboxylase modified with 6 half-cystine residues per molecule has the same Hill coefficient, K_a and V, as the native enzyme.

The effects of ATP, acetyl-CoA, and oxalacetate, on the rates of inactivation of pyruvate carboxylase by sulfhydryl reagents depend upon the anion present, the

Abbreviations: PHMB, *p*-hydroxymercuribenzoate; DTNB, 5,5′-dithiobis-(2-nitrobenzoate).

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reagent used, and the phase of inactivation considered. The dependence of the protection on acetyl-CoA concentration showed that the protection by acetyl-CoA is a cooperative phenomenon. This cooperativity was interpreted as evidence of a cooperative binding of acetyl-CoA to pyruvate carboxylase, and of a sequential change in the conformation of the enzyme in response to the binding of acetyl-CoA.

INTRODUCTION

Chicken pyruvate carboxylase (pyruvate:CO_2 ligase (ADP), EC 6.4.1.1) is a tetrameric enzyme with a molecular weight of 655 000* and about 55 sulfhydryl groups[1,2]. The tetramer can be dissociated in the cold[1-3] and by reaction with *p*-hydroxymercuribenzoate (PHMB), cystine and some other sulfhydryl-group reagents[4]. The enzyme is protected against inactivation and dissociation at 0–5 °C by 0.1 M potassium phosphate, or by higher concentrations of KCl, K_2SO_4 or $(NH_4)_2SO_4$, and by acetyl-CoA, ATP or oxaloacetate[3]. Besides the effects on the cold inactivation, ATP and sulfate ions protect against inactivation by avidin[5,6], and sulfate ions increase the K_a for acetyl-CoA[7].

Pyruvate carboxylase from chicken liver has an absolute requirement[8] for an acyl-CoA in the catalysis of Reaction 1. Acetyl-CoA is the most effective activator of all acyl-CoA

$$\text{Pyruvate} + \text{Mg–ATP} + HCO_3^- \xrightleftharpoons{Mg^{2+},\ \text{acetyl-CoA}} \text{oxaloacetate} + \text{Mg–ADP} + P_i \qquad (1)$$

compounds studied[7]. The enzymatic activity of pyruvate carboxylase shows a sigmoidal dependence on acetyl-CoA concentration[9], which was interpreted as additional evidence for an allosteric mode of interaction for acetyl-CoA.

In a previous paper[4], the effects of different sulfhydryl reagents on the activity and quaternary structure of chicken pyruvate carboxylase were studied in a buffer solution containing sulfate and phosphate ions. The inactivation by certain sylfhydryl reagents was found to be accompanied by dissociation of the tetrameric enzyme to monomers. The present paper shows that the rates and patterns of inactivation by *N*-ethylmaleimide and cystine, and the reactivity of the sulfhydryl groups of pyruvate carboxylase, depend markedly on the salt composition of the buffer solution used. The effects of ATP, acetyl-CoA and oxaloacetate, on these inactivations are also determined by the salt present. The interaction of partially modified pyruvate carboxylase preparations with acetyl-CoA are found to be different from that of native enzyme. The results are discussed in relation to different conformations of the native enzyme, allosteric interactions and the mechanism of inactivation of pyruvate carboxylase.

MATERIAL AND METHODS

Pyruvate carboxylase from chicken liver was purified through stage 5 of the procedure described by Scrutton *et al.*[10]. When a highly purified enzyme was needed, stage 5 preparation was subjected to chromatography on a column of DEAE-

* Recent work by M. F. Utter and collaborators (personal communication) indicates that the molecular weight of chicken pyruvate carboxylase may be considerably lower than previously reported[1].

Sephadex A-50 using an $(NH_4)_2SO_4$ gradient for the elution (M. C. Scrutton, personal communication). The enzyme was stored at 0–5 °C (protein concentration about 20 mg/ml) in 1.6 M sucrose, 0.1 M Tris–H_2SO_4 (pH 6.7), and 0.06 M $(NH_4)_2SO_4$. This preparation was equilibrated with the required solution by passage through Sephadex G-25. For most of the experiments reported in this paper, one of the three following buffers was used. Phosphate buffer solution contained 1 mM EDTA, and 211 mM potassium phosphate (pH 7.2); Sulfate buffer solution contained 164 mM $(NH_4)_2SO_4$, 1 mM EDTA, and 10 mM potassium phosphate (pH 7.2); and Chloride buffer solution contained 490 mM KCl, 1 mM EDTA, and 10 mM potassium phosphate (pH 7.2). The ionic strength of all three buffers was identical (I 0.525).

Pyruvate carboxylase was assayed spectrophotometrically in the direction of CO_2 fixation with the coupled malate dehydrogenase system, as described previously[10]. Either Tris–H_2SO_4 or Tris–HCl was used as a buffer. Specific activities are expressed as μmoles of oxaloacetate formed per min per mg of protein at 25 °C. Protein concentration was determined by the method of Warburg and Christian[11]. The incubations of the enzyme with the sulfhydryl reagents were carried out at room temperature.

The reaction of *N*-ethylmaleimide with pyruvate carboxylase was followed by measuring the decrease in absorbance at 300 nm[12]. The reaction of cystine with pyruvate carboxylase was followed by determining the incorporation of radioactivity into the protein upon incubation with uniformly labeled L-[^{14}C]cystine. The reaction was stopped with 5% trichloroacetic acid, and the precipitated protein was separated from the solution and washed with 5% trichloroacetic acid on a disc of filter paper (Whatman No. 42). The radioactivity retained by the filter paper was determined with a Mark I Liquid Scintillation Computer System (Nuclear Chicago Corporation).

N-Ethylmaleimide and cystine solutions were prepared immediately prior to use. *N*-Ethylmaleimide concentration was determined from the absorbance at 300 nm[12]. Because of the low solubility of cystine at neutral pH, it was dissolved at a higher or lower pH and the solution readjusted to pH 7.2 prior to its addition to pyruvate carboxylase.

Acetyl-CoA was prepared from coenzyme A (P–L Laboratories) and acetic anhydride as described by Stadtman[13], and its concentration was determined with citrate synthase (EC 4.1.3.7) according to the method of Ochoa[14]. L-[^{14}C]Cystine of a specific activity of 271 mCi/mmole was obtained in 0.1 M HCl solution from New England Nuclear. Ammonium sulfate was recrystallized twice from 1 mM EDTA solution. Other chemicals were reagent grade materials obtained from commercial sources.

Ultracentrifugal examinations were carried out in a Beckman Spinco Analytical Ultracentrifuge (Model E), using the AN-E rotor, 30-mm double sector cells and at temperatures near 21 °C. The preparation for study was placed in a cell with a wedge window, and the control enzyme in the standard cell to facilitate direct comparison.

RESULTS

Inactivation by N-ethylmaleimide and cystine in the presence of different inorganic salts

N-Ethylmaleimide was shown to inactivate pyruvate carboxylase in two steps in a buffer solution containing $(NH_4)_2SO_4$ and potassium phosphate[4]. The rate and pattern of inactivation of pyruvate carboxylase by *N*-ethylmaleimide depends on the

salt composition of the buffer solution used. Fig. 1 shows that in the presence of $(NH_4)_2SO_4$ the loss of activity took place in two steps. The substitution of potassium for ammonium did not produce any significant change in the pattern of inactivation. When phosphate was substituted for sulfate, the pattern of inactivation was essentially the same (*cf.* Fig. 4). The first phase of inactivation in phosphate or sulfate occurs with the formation of a partially active enzyme*. In contrast, in the presence of KCl at a concentration required to give the same ionic strength as sulfate, the inactivation followed pseudo-first-order kinetics with time (Fig. 1). Phosphate, at the concentration used to buffer the KCl solution (10 mM) apparently did not have any significant effect on the type of inactivation.

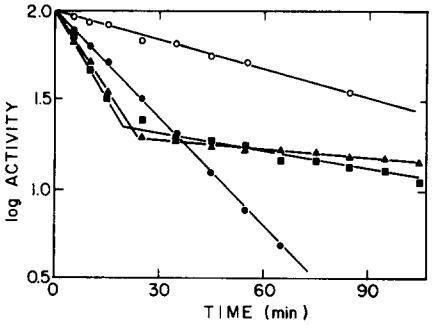


Fig. 1. Inactivation by *N*-ethylmaleimide in buffers of different salt composition. Pyruvate carboxylase (spec. act. 10.4–15.8; 0.29 mg/ml) was incubated with 0.2 mM *N*-ethylmaleimide in different buffer solutions, all of which contained 0.15 M KCl, 0.01 M potassium phosphate and 1 mM EDTA, and had a pH equal to 7.2. In addition to the common components, the following salts were included: ■, 0.06 M $(NH_4)_2SO_4$; ▲, 0.06 M K_2SO_4; ●, 0.18 M KCl; and ○, 0.65 M KCl. All enzymatic activities were determined on aliquots of the incubation mixtures at the indicated times of incubation. Activities are expressed as percentages of the activity of the corresponding control without inhibitor.

The decrease in enzymatic activity that takes place upon partial modification of pyruvate carboxylase by *N*-ethylmaleimide in Sulfate buffer solution, could be due to either a change in the K_m values for the substrates or to an effect on V. Therefore, the apparent K_m values for Mg–ATP, free Mg^{2+}, bicarbonate, and pyruvate of the modified and the native enzymes were determined. Table I shows that there are no large differences, if any, between the K_m values obtained with the modified enzyme and those obtained with the native enzyme. These apparent K_m values agree reasonably well with those determined previously for the native enzyme[7,10].

The V of *N*-ethylmaleimide-32-pyruvate carboxylase (sulfate) is about 50% of that of the native enzyme (*cf.* Table V), as determined from the position of the "break" in the semilogarithmic plot of enzymatic activity *versus* time of incubation with *N*-ethylmaleimide, using saturating concentrations of substrates and acetyl-CoA in the activity assay mixture.

* The fact that the break in the curve of inactivation does not occur at the same relative activity with respect to the control in all experiments is a reflection of the different dependences of activity on acetyl-CoA concentration in the native and the modified enzyme and the use of Tris-H_2SO_4 in the assay mixture of some experiments, changing in this way the effective acetyl-CoA concentration to nonsaturating levels for the modified enzyme.

TABLE I

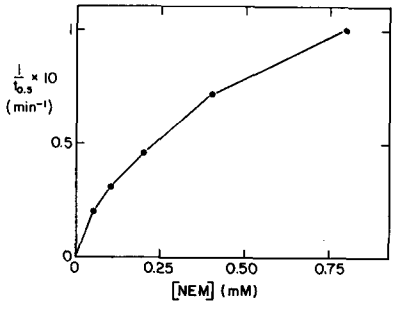
MICHAELIS CONSTANTS FOR SUBSTRATES OF PYRUVATE CARBOXYLASE

K_m values were obtained from primary plots of $1/v$ *versus* $1/[S]$, except for the K_m for bicarbonate. The K_m for Mg–ATP was obtained in the presence of 4 mM free Mg^{2+}, and that for free Mg^{2+} in the presence of 2 mM Mg–ATP. Because of the difficulty of eliminating bicarbonate from the assay mixture, the K_m value was obtained by estimating the endogenous bicarbonate. Three enzymatic activities were determined: in the absence of added bicarbonate, and after addition of intermediate and saturating concentrations of bicarbonate. The K_m was obtained from the three values of the enzymatic activity, and the two concentrations of bicarbonate added to the assay mixture, using the Michaelis–Menten relationship. The endogenous bicarbonate concentration was estimated to range from 0.23 to 0.42 mM, under the conditions of the assay.

Substrate	*Apparent K_m (mM)*	
	*N-Ethylmaleimide-32-pyruvate carboxylase**	*Native pyruvate carboxylase*
Mg–ATP	0.25	0.33
Mg^{2+}	0.30	0.30
Bicarbonate	1.5	1.1
Pyruvate	0.51	0.48

* *N*-Ethylmaleimide-32-pyruvate carboxylase (sulfate), obtained as described in Table V.

The dependence of the rate of inactivation on *N*-ethylmaleimide concentration in the Chloride buffer solution (see Material and Methods) is shown in Fig. 2. At all concentrations of *N*-ethylmaleimide, the inactivation followed pseudo-first-order kinetics with respect to time. However, the dependency of the rate of inactivation on reagent concentration was non-linear. The plot of reaction rate *versus* *N*-ethylmaleimide concentration produced downward curvature (Fig. 2), indicating that a change in the rate-limiting step of the inactivation occurs as the concentration of *N*-ethylmaleimide is varied[15].



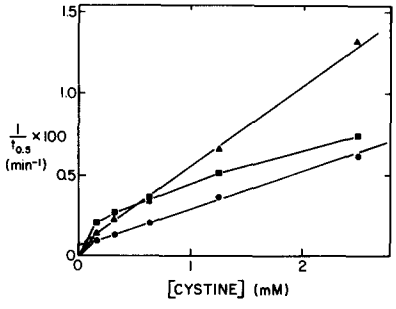


Fig. 2. Dependence of the rate of inactivation by *N*-ethylmaleimide in Chloride buffer solution on the reagent concentration. Pyruvate carboxylase (spec. act. 15.6; 0.27 mg/ml) was incubated with different concentrations of *N*-ethylmaleimide, in Chloride buffer solution. At different times after the addition of *N*-ethylmaleimide, enzymatic activities were determined on aliquots of the incubation mixtures. The reciprocal of the half-time of inactivation, obtained from linear semi-logarithmic plots is plotted *versus* *N*-ethylmaleimide concentration.

Fig. 3. Dependence of the rates of inactivation by cystine in the presence of different salts, on the reagent concentration. Pyruvate carboxylase (spec. act. 15.5; 0.3 mg/ml) was incubated with cystine in buffers of different salt composition (●, Phosphate buffer solution; ▲, Sulfate buffer solution; ■, Chloride buffer solution). At different times after the addition of cystine, enzymatic activities were determined on aliquots of the incubation mixtures. The reciprocal of the half-time of inactivation obtained from linear semi-logarithmic plots is plotted *versus* cystine concentration.

In all three buffer solutions, cystine produced pseudo-first-order inactivation with time. The lines consistently extrapolated to higher than 100% activity at zero time, indicating that a lag occurs before the onset of inactivation. The existence of such a lag can be explained by assuming the presence of two different classes of sulfhydryl groups[16]. The modification of the most reactive class might have little or no immediate effect on the activity, but would be necessary for the reaction of the second class, which is accompanied by the loss of activity. The dependence of the rate of inactivation on cystine concentration (Fig. 3) is consistent with this interpretation. In each of the three solutions, the rate of inactivation varies linearly with cystine concentration, as expected if each reaction were first order with respect to cystine concentration. However, the lines extrapolate to the ordinate at values well above zero. These results can be interpreted if we assume that the enzyme has at least one very reactive sulfhydryl group which reacts to produce a modified enzyme. This modified enzyme is active but less stable than the native enzyme and inactivates slowly, without further binding of reagent, following first-order kinetics. It seems likely that this reactive class of sulfhydryls corresponds to the first of the two classes postulated to explain the lag before inactivation.

Sedimentation patterns of the inactivated preparations

The study of the sedimentation velocity patterns of pyruvate carboxylase inactivated by each of the two SH-group reagents, in all three buffer solutions chosen, was important in the construction of a unified scheme showing the effect of modification on the activity and quaternary structure of the enzyme. The first step of inactivation by *N*-ethylmaleimide in both Phosphate and Sulfate buffer solutions took place without any significant change in the sedimentation pattern, in agreement with previous results using a buffer solution with both phosphate and sulfate[4]. During the second step of inactivation there was a disappearance of tetramers and a formation of high molecular weight aggregates. No monomers were clearly detected, although there was an indication of their presence in very small amounts. In the Chloride buffer solution, the decrease in tetramers was accompanied by the appearance of a small proportion of monomers.

The inactivation by cystine was accompanied in all three buffer solutions by a decrease in the amount of tetramers and a formation of monomers. However, the relation between the extent of dissociation and that of inactivation was not the same in all cases, indicating the presence in the preparations of different relative amounts of inactive tetramers.

Effects of ATP, acetyl-CoA and oxaloacetate on the inactivations by N-ethylmaleimide and cystine

The ratio of the rates of inactivation in the presence and in the absence of the compound being tested, was determined from the straight-line steps obtained in a plot of the logarithm of the enzymatic activity *versus* the time of incubation. Typical data are shown in Fig. 4 for acetyl-CoA and oxalacetate. In some cases, only a qualitative estimate of the effect was made. In no case did the compounds tested change the pattern of inactivation (two-steps *versus* pseudo-first-order). Nor did they have any apparent effect on the stability of pyruvate carboxylase in the absence of *N*-ethylmaleimide during the time of the experiment, except for oxaloacetate which caused a

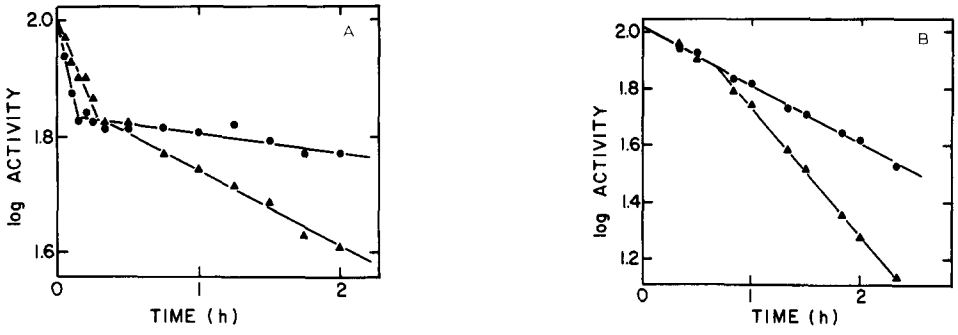


Fig. 4. Protection by ligands. (A) Effect of acetyl-CoA on the inactivation by *N*-ethylmaleimide in Phosphate buffer solution. Pyruvate carboxylase (spec. act. 21.7; 0.26 mg/ml) was incubated with *N*-ethylmaleimide (0.2 mM), in the presence (▲) and absence (●) of acetyl-CoA (1 mM). The enzymatic activity was determined on aliquots of the incubation mixtures at different times of incubation. (B) Effect of oxaloacetate on the inactivation by cystine in Sulfate buffer solution. Pyruvate carboxylase (spec. act. 18.0; 0.3 mg/ml) was incubated with cystine (2 mM), in the presence (▲) and absence (●) of oxaloacetate (3 mM). The enzymatic activity was determined on aliquots of the incubation mixtures at different times of incubation.

significant inactivation in the Chloride buffer solution. The rates of inactivation by *N*-ethylmaleimide in the presence of oxaloacetate were corrected for this effect.

The data for the effects of ATP, acetyl-CoA and oxaloacetate on the rates of inactivation by *N*-ethylmaleimide under different conditions are summarized in Table II. ATP had a protective effect against inactivation in the presence of chloride; however, it produced a very small protection against both phases of inactivation in sulfate, and showed no effect on the inactivation in phosphate. The latter is consistent if phosphate binds to the same site as ATP[5]. Acetyl-CoA, at a concentration of 1 mM, protected almost completely in the Chloride buffer solution. In the other two solutions, it afforded protection against the first phase of inactivation but increased the rate of the second (Fig. 4A). Oxaloacetate caused an increase in the rate of inactivation in chloride, and in phosphate and sulfate it behaved qualitatively as acetyl-CoA, *i.e.*

TABLE II

EFFECTS OF ATP, ACETYL-CoA AND OXALOACETATE ON THE INACTIVATION BY *N*-ETHYLMALEIMIDE

Pyruvate carboxylase (spec. act. 12.9–25.2; 0.3 mg/ml) was incubated with *N*-ethylmaleimide in the presence and in the absence of the compound being tested. Because of the wide range of the rate of inactivation under the different conditions, three levels of *N*-ethylmaleimide were used: $2 \cdot 10^{-5}$–$4 \cdot 10^{-5}$ M (*a*), 10^{-2} M (*b*), and $1 \cdot 10^{-4}$–$2 \cdot 10^{-4}$ M (the rest). The enzymatic activities were determined on aliquots of the incubation mixtures at different times of incubation. The velocities of inactivation were obtained from plots of log of activity *versus* time. v represents the rate of inactivation in the presence of the compound being tested, and v_1 in its absence.

Addition	v/v_1				
	Chloride	*Sulfate*		*Phosphate*	
		Phase 1 (a)	*Phase 2 (b)*	*Phase 1 (a)*	*Phase 2 (b)*
17 mM ATP	—	— [a]	—	1.0[a]	1.0[b]
4 mM ATP	0.26	<1.0	0.74[b]	—	—
1 mM acetyl-CoA	0.07	0.23	2.4	0.67	3.5
1 mM oxaloacetate	—	0.29	1.8	0.33	8.7
3 mM oxaloacetate	4.8	—	—	—	—

protecting against the first phase of inactivation and increasing the rate of the second. The effect of oxaloacetate might be due to the formation of the carboxylated enzyme, rather than to the direct binding of oxaloacetate.

The effects on the inactivation produced by cystine are shown in Table III. In chloride, the effects of the three compounds were qualitatively the same as in the case of *N*-ethylmaleimide: ATP and acetyl-CoA protected, and oxaloacetate increased the

TABLE III

EFFECTS OF ATP, ACETYL-CoA, AND OXALOACETATE ON THE INACTIVATION BY CYSTINE

Pyruvate carboxylase (spec. act. 12.9–23.7; 0.3 mg/ml) was incubated with cystine (2 mM), in the presence and absence of the compound being tested. The velocities of inactivation were obtained as indicated in Table II.

Addition	v/v_1		
	Chloride	*Sulfate*	*Phosphate*
17 mM ATP	—	—	1.0
4 mM ATP	0.25	0.73	—
1 mM acetyl-CoA	<0.03	0.14	0.35
3 mM oxaloacetate	9.0	2.0	1.0

rate of inactivation. In the other two solutions, ATP produced the same effects as in the *N*-ethylmaleimide inactivations, with protection in sulfate and no effect in phosphate solution. Acetyl-CoA showed a protective effect towards cystine inactivation, as in the first phase of the inactivation by *N*-ethylmaleimide. In the Sulfate buffer solution, oxaloacetate increased the rate of cystine inactivation, as in the second phase of the *N*-ethylmaleimide inactivation; however, after the addition of cystine there was a period of time in which no increase in the rate of inactivation took place (Fig. 4B). The same behavior with oxaloacetate was observed in Chloride buffer solution. The shape of the curve of inactivation in the presence of oxaloacetate suggests that this compound protected the enzyme against the modification that takes place during the lag preceding inactivation. Oxaloacetate did not have any effect in phosphate.

Pyruvate was tested under a variety of conditions, but it showed no effect in any case.

Concentration dependence of the protection by acetyl-CoA and ATP against inactivation

Acetyl-CoA protects pyruvate carboxylase completely against inactivation by *N*-ethylmaleimide or cystine in Chloride buffer solution. The plot of relative protection (rate of inactivation in the absence of ligand, v_1, *minus* the rate of inactivation in the presence of ligand, v) *versus* acetyl-CoA concentration produces a sigmoidal curve, in both cases (Fig. 5), indicating that the protection by acetyl-CoA is a cooperative phenomenon. However, the degree of cooperativity is not the same in the two cases. The interaction coefficient for protection, n, is 2.2 in the inactivation by *N*-ethylmaleimide, and 2.8 in the inactivation by cystine (Table IV). *N*-Ethylmaleimide and cystine cause an initial and rapid partial modification of pyruvate carboxylase, producing *N*-ethylmaleimide-16-pyruvate carboxylase (chloride), and cystine-6-pyruvate carboxylase (chloride), with full enzymatic activity. Therefore, acetyl-CoA is most likely protecting against the inactivation of the partially modified enzymes instead of

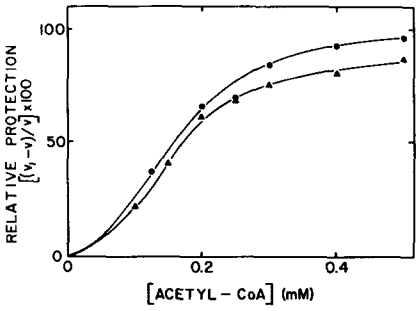


Fig. 5. Dependence of the protection by acetyl-CoA against inactivation by *N*-ethylmaleimide and cystine on acetyl-CoA concentration. Pyruvate carboxylase (spec. act. 14.6 units/mg; 0.28 mg/ml) was incubated with 0.1 mM *N*-ethylmaleimide (▲), and 2 mM L-cystine (●), in the presence of different concentrations of acetyl-CoA, in the Chloride buffer solution. For other experimental conditions, see Material and Methods.

the native enzyme. Acetyl-CoA also affords complete protection against the inactivation by cystine in Sulfate buffer solution. In this case, a value of 1.8 was obtained for the interaction coefficient (Table IV). The value of the protection constant, K_p, is also given in this table. The K_p for acetyl-CoA in sulfate is about 5 times higher than in chloride, as is expected from the competitive effect of sulfate on the K_a for acetyl-CoA[7].

TABLE IV

PROTECTION BY ACETYL-CoA AND ATP AGAINST THE INACTIVATION BY *N*-ETHYLMALEIMIDE AND CYSTINE

The experiments were conducted as those described in Fig. 5.

Reagent	*Buffer solution*	*Acetyl-CoA*		*ATP*	
		K_p* (μM)	n**	K_p* (μM)	n**
N-Ethylmaleimide	Chloride	180	2.2	460	1.1
Cystine	Chloride	170	2.8	520	1.0
Cystine	Sulfate	840	1.8	Not determined	

* K_p, protection constant is the concentration of ligand that produces half-maximal protection, $v = (v_1 - v_2)/2$. Where v, v_1 and v_2 are the rates of inactivation in the presence of ligand, in the absence of ligand, and at saturating ligand, respectively.

** n is the interaction coefficient obtained from the slope of a plot of log $(v_1 - v)/(v - v_2)$ *versus* log [ligand].

ATP gives only partial protection against the inactivation of pyruvate carboxylase by *N*-ethylmaleimide or cystine in Chloride buffer solution. The dependence of $(v_1 - v)$ on ATP concentration is of a hyperbolic type, for the inactivation by *N*-ethylmaleimide. Using the value obtained by extrapolation of the data to infinite concentration of ATP in a reciprocal plot, $1/(v_1 - v)$ *versus* $1/[ATP]$, for v_2 (Table IV), the value obtained for n was 1.1, close to 1.0 expected from a Michaelis–Menten relationship. The same results were obtained in the inactivation by cystine (Table IV). The value of the protection constant for ATP is high, when compared with the value for the K_p previously obtained by Scrutton and Utter[1] from the study of the protection by ATP against the inactivation by avidin.

Correlation of the binding of N-ethylmaleimide and cystine with the loss of enzymatic activity

Fig. 6A shows the binding of *N*-ethylmaleimide and the increased inactivation with time for a preparation of pyruvate carboxylase in Sulfate buffer solution. The *N*-ethylmaleimide concentration was low enough so that the rate of the second step of inactivation was negligibly small. The formation of a partially active enzyme, that takes place during the first step of inactivation, is correlated with the binding of about 30 sulfhydryl groups per tetramer*. During the second step of inactivation, there is a precipitation of the high molecular weight aggregates that interferes with the spectrophotometric determination of the binding of *N*-ethylmaleimide. The results obtained in the Phosphate buffer solution are similar to those obtained in the Sulfate buffer solution.

Because of the high reactivity of the sulfhydryls that bind to *N*-ethylmaleimide during the first step of inactivation in the presence of sulfate or phosphate ions, it is possible to titrate the enzymatic activity with *N*-ethylmaleimide. The enzyme was incubated with *N*-ethylmaleimide, at different low molar ratios (0–50) of *N*-ethylmaleimide to tetramer, and the specific activities were determined after 18 h of incubation. A break in the curve of activity *versus* molar ratio of *N*-ethylmaleimide takes place at a molar ratio of *N*-ethylmaleimide to enzyme equal to 32 and corresponds to the end of the first phase of inactivation. This number is close to the number of SH groups that were directly determined spectrophotometrically to have reacted at this point (Fig. 6A). The fact that the relative activity at the breaking point is close to that obtained in inactivation-rate experiments at short times indicates that the modified enzyme is fairly stable, under the conditions of the experiment. This was used as a method to prepare *N*-ethylmaleimide-32-pyruvate carboxylase (*cf.* Table V). The relative activities of the preparations incubated with *N*-ethylmaleimide at a molar ratio

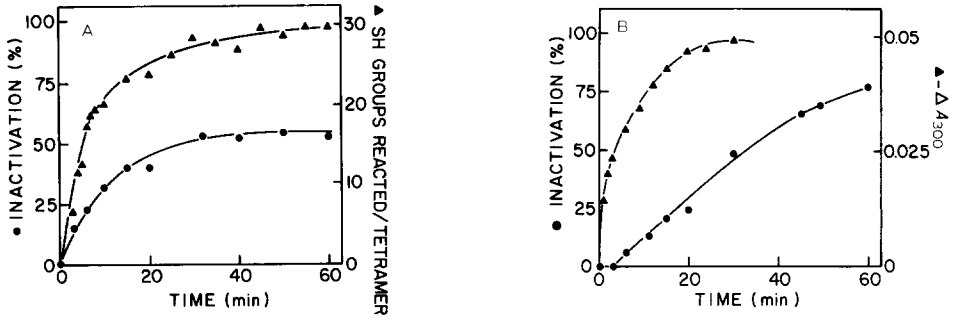


Fig. 6. Spectrophotometric titration with *N*-ethylmaleimide. The reaction was followed by measuring the decrease in absorbance at 300 nm with time. Immediately after the titration was completed, another incubation mixture was prepared exactly as the one that had been used for the titration, and the enzymatic activity was measured at different times of incubation. (A) Sulfate buffer solution (pyruvate carboxylase: spec. act. 34.6, 1.2 mg/ml; *N*-ethylmaleimide: 0.125 mM). (B) Chloride buffer solution. (pyruvate carboxylase: spec. act. 29.2, 1.41 mg/ml; *N*-ethylmaleimide 0.3 mM). After about 25 min the $\Delta A_{300\ nm}$ increased due to a precipitation of high molecular weight aggregates that interfered with the determination.

* Although *N*-ethylmaleimide may react with groups other than sulfhydryl (ref. 17), it seems safe to conclude that, under the conditions of these experiments, reaction with sulfhydryl groups predominates.

higher than 32 continued to decrease after the first 18 h of incubation, indicating that at this time the reaction with *N*-ethylmaleimide had not yet reached completion.

The spectrophotometric titration with *N*-ethylmaleimide in the Chloride buffer solution (Fig. 6B) showed an initial modification of about 16 SH groups per tetramer without loss of activity, after which inactivation parallels the binding of all the remaining sulfhydryl groups.

The extent of inactivation of pyruvate carboxylase by L-cystine, and the number of SH groups modified at different times in the Sulfate buffer solution are shown in Fig. 7A. The lag before inactivation is accompanied by the reaction with cystine of 4–6 very reactive sulfhydryls, while the inactivation of the enzyme corresponds to the modification of about 16 additional groups. Since the inactivation is first order with time (Fig. 3) during the latter stage, it is most likely that these 16 sulfhydryl groups

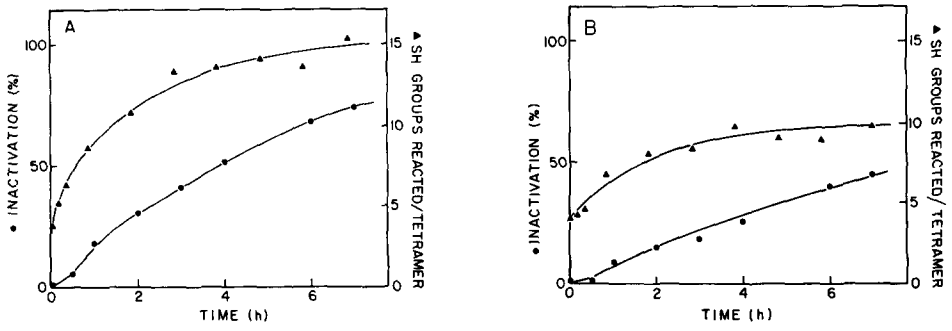


Fig. 7. Titration with L-[^{14}C]cystine in sulfate buffer solution in the presence (B) and absence (A) of acetyl-CoA. The reaction mixtures contained, in addition to Sulfate buffer solution: pyruvate carboxylase (0.61 mg/ml; spec. act. 24 units/mg), and L-[^{14}C]cystine (0.825 mM; 2.4 mCi/mmole); 0.8 mM acetyl-CoA was included in B. At the times indicated in the figure, 5% trichloroacetic acid was added to aliquots of the reaction mixtures, and the radioactivity of the precipitated protein was determined as indicated under Material and Methods. The enzymatic activity was determined on aliquots of the reaction mixtures at different times of incubation.

are reacting in an "all or none" manner. Similar numbers of groups react in the presence of 0.8 mM acetyl-CoA (Fig. 7B), but at a slower rate. Thus, acetyl-CoA protects the enzyme against inactivation by cystine by lowering the reactivity of the sulfhydryl groups, and not by preventing a conformational change of the already modified enzyme. A similar relationship between the reaction of the SH groups of pyruvate carboxylase and its inactivation by cystine was obtained in the Chloride buffer solution.

Dependence of the enzymatic activity of different modified pyruvate carboxylases on acetyl-CoA concentration

The forms of pyruvate carboxylase obtained by partial modification with *N*-ethylmaleimide, in the three standard buffer solutions, present kinetics of activation by acetyl-CoA different from that of the native enzyme. Fig. 8 is a plot of enzymatic activity *versus* acetyl-CoA concentration for *N*-ethylmaleimide-32-pyruvate carboxylase (sulfate) and the native enzyme. In both cases, the shape of the curve is sigmoidal, but the degree of sigmoidicity and the activation constant for acetyl-CoA are not the same. When these data are plotted in a Hill representation[18], a Hill coefficient equal

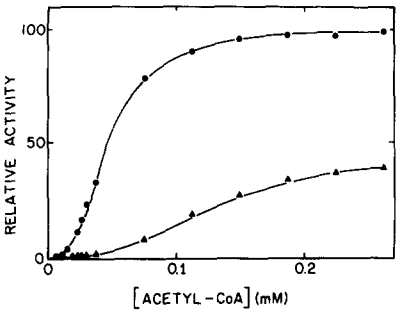


Fig. 8. Dependence of enzymatic activity on acetyl-CoA concentration. The enzymatic activities, v, were determined in the presence of 80 mM K_2SO_4, in order to minimize the relative effect on the K_a of the low amounts of sulfate, 1.6 mM or less, added in some cases with the enzyme to the assay mixture. ●—●, native pyruvate carboxylase; ▲—▲, *N*-ethylmaleimide-32-pyruvate carboxylase (sulfate).

to 2.2 is obtained for *N*-ethylmaleimide-32-pyruvate carboxylase (sulfate), in contrast with the value 2.7 for the native enzyme. This change in the Hill coefficient was accompanied by an increase in the K_a from 47 μM for the native enzyme to 140 μM for *N*-ethylmaleimide-32-pyruvate carboxylase (sulfate). The values for K_a, n and V, obtained with the enzyme modified by *N*-ethylmaleimide in Phosphate buffer solution, *N*-ethylmaleimide-32-pyruvate carboxylase (phosphate), were similar to those of *N*-ethylmaleimide-32-pyruvate carboxylase (sulfate) (Table V) and are, therefore, probably the same molecular species. The enzyme modified by *N*-ethylmaleimide in Chloride buffer solution, *N*-ethylmaleimide-16-pyruvate carboxylase (chloride), pre-

TABLE V

DEPENDENCE OF ENZYMATIC ACTIVITY ON ACETYL-CoA CONCENTRATION FOR DIFFERENT MODIFIED PYRUVATE CARBOXYLASES

The experiments were conducted as described in Fig. 8. The modified enzymes NEM-32-PC (phosphate) and NEM-32-PC (sulfate) were prepared by incubating native pyruvate carboxylase for 6-16 hours with NEM, at a molar ratio of NEM to enzyme equal to 32, in "Phosphate" and "Sulfate Buffer Solution", respectively. NEM-16-PC (chloride) was obtained after incubation in "Chloride Buffer Solution" for 6–8 hours, with a molar ratio of NEM to enzyme equal to 16. The three pyruvate carboxylases modified by cystine were obtained by incubation with 60 μM cystine for 6–8 hours in: "Phosphate Buffer Solution", Cystine-PC (phosphate); "Sulfate Buffer Solution", Cystine-6-PC (sulfate); and "Chloride Buffer Solution", Cystine-6-PC (chloride). In all cases, the concentration of pyruvate carboxylase in the incubation mixture was 0.3–0.6 mg/ml.

Enzyme	K_a* (μM)	n**	V*** (*relative value*)
Native pyruvate carboxylase	47	2.7	100
N-Ethylmaleimide-32-pyruvate carboxylase (phosphate)	130	2.3	50
N-Ethylmaleimide-32-pyruvate carboxylase (sulfate)	140	2.2	50
N-Ethylmaleimide-16-pyruvate carboxylase (chloride)	58	2.2	100
Cystine-pyruvate carboxylase (phosphate)	47	2.7	100
Cystine-6-pyruvate carboxylase (sulfate)	49	2.7	100
Cystine-6-pyruvate carboxylase (chloride)	44	2.9	100

* K_a, activation constant, is the concentration of acetyl-CoA that produces half-maximal activity.

** n is the Hill coefficient[18].

*** V was obtained from the kinetics of inactivation.

sented a low value for the Hill coefficient similar to those of *N*-ethylmaleimide-32-pyruvate carboxylase (phosphate, or sulfate), but no change in V, and only a small increase in K_a were observed when compared with the native enzyme (Table V). In contrast with the forms of pyruvate carboxylase obtained by modification with *N*-ethylmaleimide, those modified by cystine, *i.e.* cystine-pyruvate carboxylase (phosphate), cystine-6-pyruvate carboxylase (sulfate), and cystine-6-pyruvate carboxylase (chloride), appeared to have identical properties and showed the same values for K_a, n and V, as the native enzyme. The protection constants for acetyl-CoA (Table IV) were much higher than the corresponding activation constants (Table V). However, the different ligands present, enzyme concentration, or ionic strength could produce the differences observed in the two constants.

DISCUSSION

The different reactivities of the SH groups of pyruvate carboxylase and the different patterns and rates of inactivation in the presence of phosphate, sulfate, or chloride, indicate that the conformation of the enzyme is affected by the three anions in different ways. Phosphate is a product of the CO_2-fixation reaction and is known to bind to the free enzyme at the active site. Sulfate is a competitive inhibitor with respect to acetyl-CoA[7], and thus presumably can bind at the allosteric-modifier site. Chloride, in all previous studies, has not been implicated in any way as binding to the enzyme at any specific site. It has been shown that phosphate protects the enzyme against cold inactivation[3], and sulfate against the inactivation by avidin[6]. These facts support our interpretation of the effects of these anions on the conformation of the native enzyme.

The process that accompanies modification of pyruvate carboxylase by sulfhydryl-group reagents is summarized in the tentative scheme of Fig. 9. Under all the

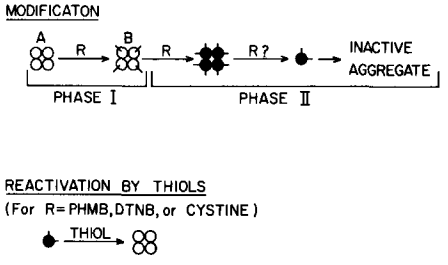


Fig. 9. Scheme showing the processes that accompany modification of pyruvate carboxylase by SH-group reagents, and its reactivation by simple thiols. ○ represents an active form of the enzyme; ● represents an inactive form; and R represents an SH-group reagent. The lines coming out of the representations of the enzyme signify the binding of reagent.

conditions studied in this paper, two phases in the modification of the enzyme can be distinguished. The number of sulfhydryls that react during each phase varies with the buffer solution and the reagent used, as is summarized in Table VI. During the first phase of modification, between 4 and 32 sulfhydryls react, and this reaction takes place with either partial (*N*-ethylmaleimide in Phosphate or Sulfate buffer solutions) or no inactivation, and with no gross change in the sedimentation properties of the enzyme (species designated as B in Fig. 9). In some cases there is an alteration in the kinetic

TABLE VI

NUMBER OF SULFHYDRYL GROUPS REACTED PER MOLECULE OF PYRUVATE CARBOXYLASE DURING THE TWO PHASES OF MODIFICATION

A value for the molecular weight lower than 655 000 would affect the calculated number of SH-groups per tetramer that react under each of the considered conditions. However, the relationship among these numbers would remain unchanged (*cf.* footnote to introduction).

Reagent	*Buffer solution*	*Phase I*	*Phase II*
N-Ethylmaleimide	Phosphate	30–32	Not determined
	Sulfate	30–32	Not determined
	Chloride	16	39
L-Cystine	Sulfate	4–6	16
	Chloride	4–6	16

behavior with respect to acetyl-CoA (Table V). The second phase of modification proceeds with reaction of an additional 16 or 39 sulfhydryls of the enzyme under the conditions in which they were measured, and is accompanied by complete inactivation and loss of the tetrameric structure. There is some evidence, although not conclusive in all cases, indicating the initial formation of an inactive tetramer during this phase. The inactive tetramer probably breaks down to its constituent monomers before irregular aggregation takes place, although the direct formation of aggregates from the modified tetramers can not be excluded.

In the presence of phosphate or sulfate, about 32 of the 55 SH groups of the enzyme react with *N*-ethylmaleimide without a complete loss of activity, indicating that the structure required for catalysis is not affected critically by the modification of such a large number of groups. In contrast, the total number of groups that react with cystine during inactivation is only about 20, less than half of the total sulfhydryls. The greater stability of the modified monomers obtained with cystine, as compared to those obtained with *N*-ethylmaleimide, might be due to the lower degree of modification attained with cystine.

Although cystine-6-pyruvate carboxylase did not show any changes in the kinetic response to acetyl-CoA, as compared with the native enzyme, *N*-ethylmaleimide-32-pyruvate carboxylase and *N*-ethylmaleimide-16-pyruvate carboxylase both presented a significant decrease (from 2.7 to 2.2) in the value of the Hill coefficient for acetyl-CoA (Table V). This partial decrease in the Hill coefficient upon modification is unusual, and indicates that the interactions among the subunits of the enzyme have been only slightly affected. In most experiments reported in the literature, the loss of cooperativity upon modification is complete, with a Hill coefficient equal to unity. Moreover, detailed studies of the activation of chicken pyruvate carboxylase[7,9,19] have shown that, under a wide range of conditions, the Hill coefficient is a rather constant parameter, being only affected by pH (ref. 9) and by the inhibitor acetoacetyl-CoA[7]. These considerations make the partial decrease in cooperativity upon modification by *N*-ethylmaleimide of special interest.

An increase in K_a concomitant with a decrease in cooperativity, as observed with *N*-ethylmaleimide-32-pyruvate carboxylase, is a change in the opposite direction to that which is usually found and which would be predicted by most theories[20–22]. The fact that acetyl-CoA protects pyruvate carboxylase against a variety of agents that cause dissociation[3], *i.e.* cold, urea and high pH, indicates that the active species

obtained in the presence of acetyl-CoA is a more tightly associated tetramer than the inactive tetramer prior to activation. It has been concluded from kinetic data that the active site of pyruvate carboxylase is formed by two subsites, one for nucleotides and bicarbonate, and the other for α-ketoacids[7]. If two subsites from two different subunits had to interact in a heterologous association, as has already been suggested[23], catalytic activity would require a very precise arrangement of the subunits. This would be produced by the tightening effect on the structure of the enzyme brought about by acetyl-CoA. An enzyme constructed in such a way would be well suited for the very strict control of enzymatic activity that is required for chicken pyruvate carboxylase[24]. In the case of *N*-ethylmaleimide-32-pyruvate carboxylase, the chemical modification would prevent the precise arrangement of subunits needed for optimal catalytical activity, and as a consequence, would produce a decrease in V. Thus, the decrease in cooperativity, the decrease in affinity, and the decrease in V could all be related to a loosening of the quaternary structure of the enzyme.

The effects of ATP, acetyl-CoA, and oxaloacetate on the rates of inactivation by *N*-ethylmaleimide and cystine, under different conditions, are so diverse that it is difficult to include them in a simple scheme. However, such diversity shows how sensitive the enzyme conformation is to the presence of other solutes, organic and inorganic. Acetyl-CoA, and probably ATP, oxaloacetate, and the ionic strength, affect the rates of inactivation by altering the rates of modification of A and B (Fig. 9). Inorganic anions would determine the number of sulfhydryls that react in the two phases of the modification, and also the enzymatic activity of B.

The effect of substrates on the rates of inactivation in different buffer solutions, seems to indicate that the sulfhydryl(s) responsible for the inactivation is (are) not located in the active site. Even at relatively high concentrations of ATP, the protection by this compound was far from complete. If free ATP binds to the active site[5], these results are evidence in favor of the location of the critical sulfhydryl(s) away from the active site. When oxaloacetate showed an effect on the second phase of modification, the phase giving complete inactivation, it was an increase in the rate of inactivation instead of protection. Pyruvate did not have any observable effect.

The protection by acetyl-CoA against the inactivation produced by *N*-ethylmaleimide or cystine allows some deductions to be made about the binding of acetyl-CoA and its effects on the structure of the enzyme. The cooperativity involved in the protection by acetyl-CoA, as opposed to the noncooperative protection by ATP, can be interpreted either as a change in the general conformation of the enzyme that takes place upon binding of acetyl-CoA, or as related directly to the binding of acetyl-CoA. The fact that acetyl-CoA, in Phosphate or Sulfate buffer solution, increases the rate of inactivation by *N*-ethylmaleimide, instead of decreasing it, seems to exclude the possibility of a critical sulfhydryl being located at the binding site for acetyl-CoA. When cystine substitutes for *N*-ethylmaleimide, in the two mentioned buffer solutions, acetyl-CoA protects completely against inactivation. If the mechanism of inactivation is the same with both reagents, as seems to be the case, the evidence favors the critical sulfhydryl group(s) being away from the binding site for acetyl-CoA. The change in the rate of inactivation by sulfhydryl reagents upon binding of acetyl-CoA would best be interpreted as a reflection of the decrease in the reactivity of the sulfhydryl group(s) critical for inactivation, that are located elsewhere than at the binding site for acetyl-CoA. This decrease in reactivity would be produced by a change

in the environment of the sulfhydryl groups caused by a conformational change.

The cooperativity of the protection by acetyl-CoA strongly suggests that acetyl-CoA binds to pyruvate carboxylase in a cooperative way. However, other less likely mechanisms involving changes in reactivities can not be excluded. The cooperativity of the protection would indicate, in this case, a sequential change in the conformation of the enzyme upon binding of successive molecules of acetyl-CoA. Such a sequential change in conformation is in conflict with a model, like the one proposed by Monod *et al.*[22], in which acetyl-CoA would bind to one of two possible conformations of the tetramer, thus, displacing the equilibrium towards the form with higher affinity for acetyl-CoA. The sequential change in conformation might involve either symmetric or asymmetric intermediate forms, although the latter seem to be more plausible. Sequential, asymmetric changes in conformation would be consistent with the model of Koshland *et al.*[25] in which each subunit changes conformation upon binding of ligand.

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