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THE REACTIVE LYSINE RESIDUE AT THE ALLOSTERIC SITE OF SHEEP KIDNEY PYRUVATE CARBOXYLASE

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SUMMARY

Investigations have revealed that sheep kidney pyruvate carboxylase (pyruvate: CO_2 ligase (ADP), EC 6.4.1.1) possesses a lysine residue whose integrity is essential for enzymic activity. Modification of the enzyme with amino group reagents such as 1-fluoro-2,4-dinitrobenzene, trinitrobenzene sulphonic acid and potassium cyanate resulted in a loss of catalytic activity. The characterisation of the major radioactive dinitrophenyl-amino acid derivative of 1-fluoro-2,4-dinitro[^{14}C]benzene labelled enzyme as ε-dinitrophenyl-lysine confirmed the identity of the reactive residue.

Inhibition of the enzyme by 1-fluoro-2,4-dinitrobenzene and trinitrobenzene sulphonic acid was first order with respect to time and inhibitor concentration. From the kinetic data obtained, it was concluded that an average of 1 molecule of these reagents reacts with the enzyme causing inactivation. The rate of inactivation with these reagents was pH dependent, increasing with increasing pH values. The ability of the allosteric effector, acetyl-CoA, to protect the enzyme against inhibition by these reagents suggested that the ε-amino group of lysine may be involved in the enzyme–acetyl-CoA interaction. The dissociation constant for the enzyme–acetyl-CoA complex was $5.4 \cdot 10^{-4}$ M.

INTRODUCTION

Characterisation of the chemical components of the active centre of an enzyme is essential for the ultimate description of a detailed molecular mechanism of the enzyme catalysed reaction. Chemical modification of amino acid residues with reagents which react with a limited number of reactive groups in enzymes without causing denaturation, is one approach by which the chemistry of the active site may be investigated. A functional role for the modified residue(s) is suggested if the modification process affects the response characteristics of the native enzyme. In most instances, however, it is not immediately clear whether the modification has occurred

Abbreviations: FDNB, 1-fluoro-2,4-dinitrobenzene; TNBS, 2,4,6-trinitrobenzene sulphonic acid.

at the active site or whether reaction with a group or groups elsewhere in the protein has caused conformation changes leading to an inactive or desensitised enzyme.

In the chemical inhibition studies reported here with sheep kidney pyruvate carboxylase (pyruvate: CO_2 ligase (ADP), EC 6.4.1.1), evidence is presented for the existence of a lysine residue whose integrity is essential for enzymic activity.

It appears significant that many of the enzymes shown to possess lysine residues whose integrity is essential for their enzymic response involve a nucleotide as either substrate or effector molecule in the reaction mechanism. This group of enzymes includes myosin A ATPase[1], ribonuclease A[2], adenosine deaminase[3] and fructose 1,6-diphosphatase[4]. ROSEN AND ROSEN[4] have shown that two lysine residues in fructose-1,6-diphosphatase are associated with the allosteric site where interaction with the negative effector, 5'-AMP, takes place. This evidence suggests that the ε-NH_2 groups may be involved in the interaction with nucleotide moieties. The present finding that a reactive lysine residue which is essential for pyruvate carboxylase activity and which is associated with the acetyl-CoA binding site adds further support to this suggestion.

MATERIALS AND METHODS

Pyruvate carboxylase was prepared from freeze-dried sheep kidney cortical mitochondria by the method of LING AND KEECH[5]. Enzymic activity was assayed using a modification of the $^{14}CO_2^-$ fixation method first described by GAILIUSIS, RINNE AND BENEDICT[6]. Aliquots of the enzyme were incubated in reaction mixtures (total volume, 0.5 ml) containing (in μmoles): *N*-ethyl morpholine–HCl (pH 8.4), 50; sodium pyruvate, 5; $MgCl_2$, 4; ATP, 1.25; $NaH^{14}CO_3$, 5, 0.25 μC /μmole; and acetyl-CoA, 0.10. After 10 min at 30°, the reaction was stopped with 0.10 ml of a saturated solution of 2,4-dinitrophenylhydrazine in 6 M HCl. The solutions were treated with solid CO_2 to displace free $^{14}CO_2$. After centrifuging to remove denatured protein, aliquots of the protein-free supernatant were dried on one inch squares of Whatman 3MM filter paper and counted in a Packard Tricarb Scintillation Spectrometer. Each assay was counted in triplicate using the counting medium described by BOUSQUET AND CHRISTIAN[7].

1-Fluoro-2,4-dinitrobenzene (FDNB) and pronase were purchased from the California Corporation for Biochemical Research. CoA and 2,4,6-trinitrobenzene sulphonic acid (TNBS) were products of the Sigma Chemical Co., Mo., U.S.A. [^{14}C]FDNB and $Ba^{14}CO_3$ were purchased from the Radiochemical Centre, Amersham, Bucks., England. DNP-amino acid standards were products of the Mann Research Laboratories, N.Y., U.S.A. Preparation and purification of the reagents has been described[8]. $NaH^{14}CO_3$ was prepared from $Ba^{14}CO_3$ by distillation of $^{14}CO_2$ from perchloric acid into an equivalent amount of NaOH.

Labelling of the enzyme with [^{14}C]FDNB involved the addition of 2 mg of enzyme to a solution containing 100 μmoles of 2-amino, 2-methyl, 1,3-propanediol–HCl buffer (pH 8.8) and 0.1 μmole of [^{14}C]FDNB (1.06 μC). The reaction was stopped by the addition of 5% (w/v) trichloroacetic acid containing $5 \cdot 10^{-3}$ M (unlabelled) FDNB. The denatured protein was collected on an oxoid membrane held on a Millipore suction apparatus. Washing was effected with several aliquots of 10^{-2} M FDNB, then 10% (w/v) trichloroacetic acid and finally 1% (w/v) acetic acid. The washed denatured protein was dissolved in 0.1 M NH_4OH and the pH adjusted to 7.6 with formic acid.

The dinitrophenylated protein was hydrolysed by incubating with pronase for 48 h at 37°. The protein hydrolysate was then lyophilised to remove volatile materials. The residue was dissolved in a minimum quantity of glass distilled water (0.2 ml) and used for electrophoretic and chromatographic analysis.

Electrophoresis was performed on Whatman 3MM paper with either pyridine–acetic acid–water (1:10:289, v/v/v) buffer (pH 3.7) or with pyridine–acetic acid–water (400:16:3600, v/v/v) buffer (pH 6.5) on a "flat-bed" apparatus at 40 V/cm. Thin-layer chromatography was performed on Eastman silica gel K layers. The chromatograms were developed with the solvent system, *n*-propanol–34% NH_4OH (7:3, v/v) for 2 h.

RESULTS

Order of enzymic inactivation with respect to time and inhibitor concentration

Plots of the log of enzymic activity as a function of time of FDNB inactivation at various concentrations of inhibitor are linear until the loss of initial activity exceeds 60% (Fig. 1). These data indicate that the inactivation process down to 40% activity exhibits pseudo first order kinetics with respect to time at any fixed concentration of FDNB. The loss of linearity after more than 60% of initial enzymic activity has been

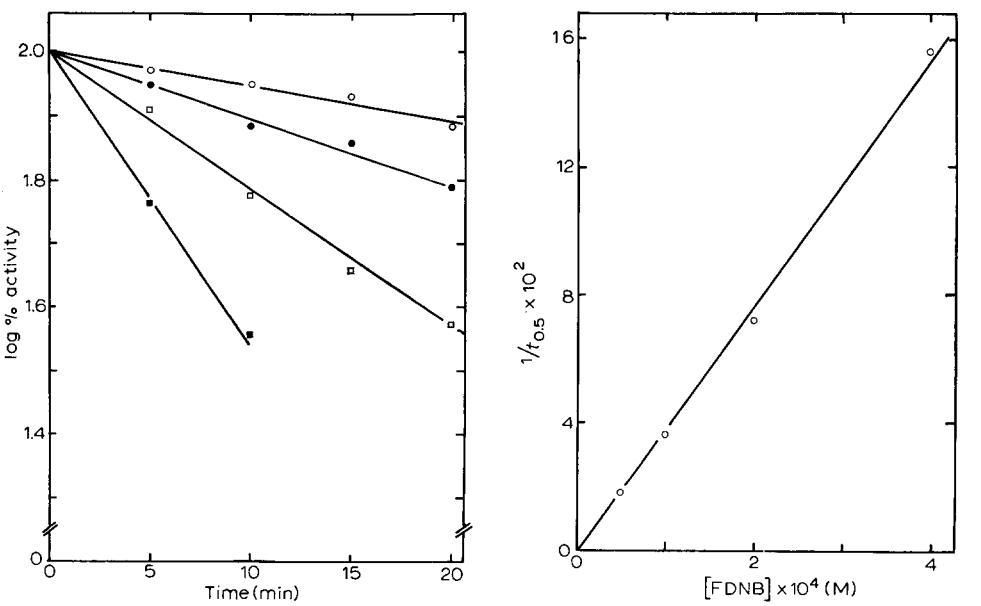


Fig. 1. Inhibition of pyruvate carboxylase with varying concentrations of FDNB. The inactivation incubation mixtures contained in a final volume of 0.3 ml, *N*-ethyl morpholine–HCl (pH 8.4) (40 μmoles), pyruvate carboxylase (0.15 unit) and varying concentrations of FDNB. Incubation was carried out at 20°. Aliquots of 0.05 ml were taken at various time intervals and assayed for residual enzymic activity. The enzyme assay mixtures contained 0.25 mg of bovine serum albumin and 0.08 μmole of DL-lysine to remove excess FDNB. The concentrations of FDNB used were $5 \cdot 10^{-5}$ M (○—○), $1 \cdot 10^{-4}$ M (●—●), $2 \cdot 10^{-4}$ M (□—□), and $4 \cdot 10^{-4}$ M (■—■).

Fig. 2. The pseudo first order kinetics of the inactivation with respect to FDNB. The half time ($t_{0.5}$) was obtained from the data shown in Fig. 1. The reciprocal of the half time was plotted against FDNB concentration.

lost indicates that, at this stage of the inactivation process, residues other than those involved in the catalytic activity are being modified by the inhibitor. The same data, when replotted (Fig. 2) so that the rate of inactivation is plotted as a function of FDNB concentration, show that the inactivation process is pseudo first order with respect to inhibitor concentration.

Furthermore, if one assumes the inactivation process as

$$E + nI \xrightarrow{k_1} EI_n \tag{1}$$

where E, I, EI_n, and k_1 are the free enzyme, inhibitor, inhibitor–enzyme complex, the number of molecules of inhibitor reacting per active site and the second order rate constant, respectively. Since the reaction between the enzyme and FDNB is essentially irreversible, the back reaction need not be considered. From Eqn. 1, the rate of the inactivation process can be written as

$$v = -\frac{dE}{dt} = k_1[E][I]^n \tag{2}$$

Although the reaction is bimolecular, it exhibits pseudo first order kinetics with the observed first order rate constant, k', equal to the true second order constant multiplied by the enzyme concentration, *i.e.*

$$k' = k_1[E] \tag{3}$$

therefore,

$$v = k'[I]^n \tag{4}$$

If the reciprocal of the half-time ($t_{0.5}$) is substituted for v and taking logs, Eqn. 4 becomes

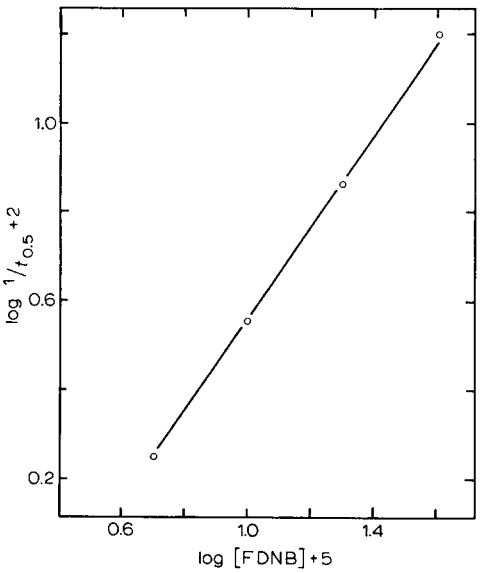
$$\log \frac{1}{t_{0.5}} = \log k' + n \log I \tag{5}$$

which is of the form of the equation of a straight line. Therefore, when $\log 1/t_{0.5}$ is plotted as a function of $\log [I]$ a straight line is obtained with slope n, *i.e.* the apparent number of molecules of inhibitor reacting to produce an inactive enzyme–inhibitor complex.

Fig. 3 presents the data of Fig. 1 replotted in this manner. The slope of the line ($n = 1.0$) indicates that an average of 1 molecule of FDNB is involved in binding to the enzyme during the inactivation process, and thus there is only one residue reacting with FDNB whose integrity is essential for enzymic activity. Using a similar type of plot, LEVY, LEBER AND RYAN[9] have concluded that at least 3 molecules of 2,4-dinitrophenol bind to myosin to bring about inactivation. Similarly, SCRUTTON AND UTTER[10] have concluded that 2 molecules of avidin are involved in the inactivation of avian liver pyruvate carboxylase and EDWARDS AND KEECH[11] have reported that 1 molecule of *N*-ethyl maleimide was bound to propionyl-CoA carboxylase causing inactivation.

Effect of pH on the rate of inactivation using FDNB

The effect of the H^+ concentration on the rate of inactivation of pyruvate carboxylase using FDNB is shown in Fig. 4. The inactivation rate increases with decreasing H^+ concentration up to pH 10.0 which indicates that the unionised ε-amino group of



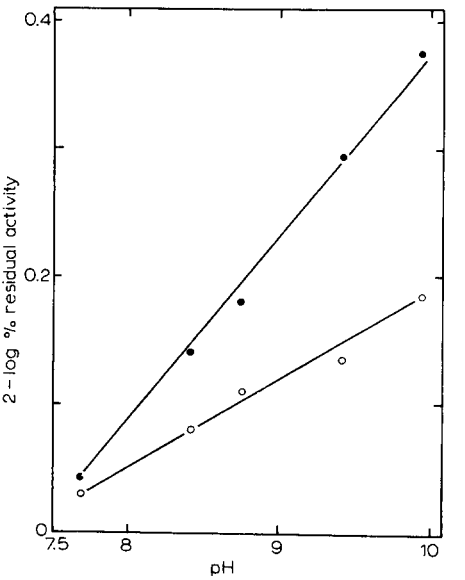


Fig. 3. Determination of the order of the reaction between pyruvate carboxylase and FDNB. The data of Fig. 1 were replotted in the form of $\log_{10} 1/t_{0.5}$ as a function of $\log_{10}$ FDNB concentration.

Fig. 4. Inhibition of enzyme with FDNB and TNBS at varying pH values. The incubation mixtures contained in a final volume of 0.30 ml, borate buffer, 4 μmoles, pyruvate carboxylase (0.15 unit) and either 0.06 μmole FDNB (○—○) or 0.2 μmole of TNBS (●—●). Borate–boric acid buffer was used in the pH region 7.5 to 9.2, and borate–NaOH buffer in the region from 9.2 to 10.0. After incubation at 20° for 5 min, aliquots were withdrawn and residual enzymic activity was measured as described for Fig. 1.

lysine reacts more readily with FDNB than does the ionised form. It was not practicable to study the process at pH values above pH 10.0 since the enzyme is not stable in this pH region. A similar pH dependence on the rate of inactivation of adenosine deaminase by FDNB was reported by RONCA, IPATA AND BAUER[3]. They showed that the FDNB was reacting with two lysine residues on the enzyme surface.

Inhibition using other chemical reagents

SANGER[12] has shown that FDNB can react with a variety of chemical groups found in proteins *viz.*, amino, sulphydryl, phenolic hydroxyl and imidazole groups. In fact FDNB has been used to implicate the involvement of one or more of these groups in the catalytic and/or allosteric sites of enzymes[2–4,13–15]. Therefore, the observed inhibition of pyruvate carboxylase activity in the presence of FDNB could involve modification of any one of the above groups. Confirmation of the presence of a reactive lysine residue was obtained by inhibiting the enzymic activity with a variety of ε-NH_2 group reagents (Table I).

The specificities of the reagents used in Table I are well documented. TNBS reacts selectively with primary amino groups, and not with phenolic, hydroxy or imidazole imino groups[16,17]. The reaction with TNBS and pyruvate carboxylase was found to be pH dependent (Fig. 4); the inactivation rate increasing with increasing pH.

TABLE I

INHIBITION OF PYRUVATE CARBOXYLASE BY AMINO GROUP REAGENTS

Experimental conditions were the same as those described in Fig. 1.

Reagent	*Concn. (M)*	$t_{0.5}$ *of inactivation (min)*	*Rate of inactivation* $(1/t_{0.5} \times 10^2)$ (min^{-1})
Potassium cyanate	$3.3 \cdot 10^{-2}$	154	0.65
TNBS	$6.7 \cdot 10^{-4}$	3.0	33.3
	$3.3 \cdot 10^{-4}$	9.3	10.7
	$1.7 \cdot 10^{-4}$	11.7	8.6
	$8.3 \cdot 10^{-5}$	22.0	4.5
FDNB	$4.0 \cdot 10^{-4}$	6.5	15.4
	$2.0 \cdot 10^{-4}$	13.8	7.2
	$1.0 \cdot 10^{-4}$	28.3	3.5
	$5.0 \cdot 10^{-5}$	56	1.8

In a separate experiment, the kinetics of the inactivation of pyruvate carboxylase with TNBS was investigated and it was found to exhibit pseudo first order kinetics with respect to both time and inhibitor concentration. Replots of the data indicated that like FDNB only 1 molecule of TNBS reacts with the enzyme to form the inactive enzyme–inhibitor complex. Inactivation of phosphorylase by potassium cyanate was shown to involve only carbamylation of the ε-NH_2 groups of lysine residues while sulphydryl groups were unaffected[18].

Coupled with the fact that there is no evidence to implicate the possible involvement of sulphydryl, hydroxyl or imidazole groups in the enzymic reaction, the inactivation of pyruvate carboxylase by these chemical reagents indicates the presence of a lysine residue in the enzyme whose integrity is essential for activity.

Identification of the [^{14}C DNP-amino acid derivative from the [^{14}C]FDNB labelled enzyme

A pronase hydrolysate of [^{14}C]FDNB labelled enzyme was subjected to electrophoretic analysis at pH 3.7 (see MATERIALS AND METHODS). Analysis of the electrophoretograms (Fig. 5) revealed a major radioactive band which had moved towards the cathode. In a similar experiment where the enzyme was protected against dinitrophenylation by acetyl-CoA (2.2 μmoles) (see below), the radioactivity of the corresponding radioactive band was reduced by 55%. In both experiments, apart from a band at the origin, no other areas were detected where the radioactivity exceeded the background. The sample lane of the electrophoretogram at pH 3.7, between 1 and 4 cm from the origin toward the cathode, was eluted with glass-distilled water, concentrated by freeze-drying and subjected to chromatographic analysis. ε-DNP-lysine was included as an internal and an external standard. External standards of *O*-DNP-tyrosine and imidazole–DNP-histidine were also used. Analysis of the chromatogram (Fig. 6) revealed a single radioactive band which was coincident with the ε-DNP-lysine spot. This suggested that the major radioactive band obtained electrophoretically was homogeneous with respect to radioactivity and co-chromatographed with ε-DNP-

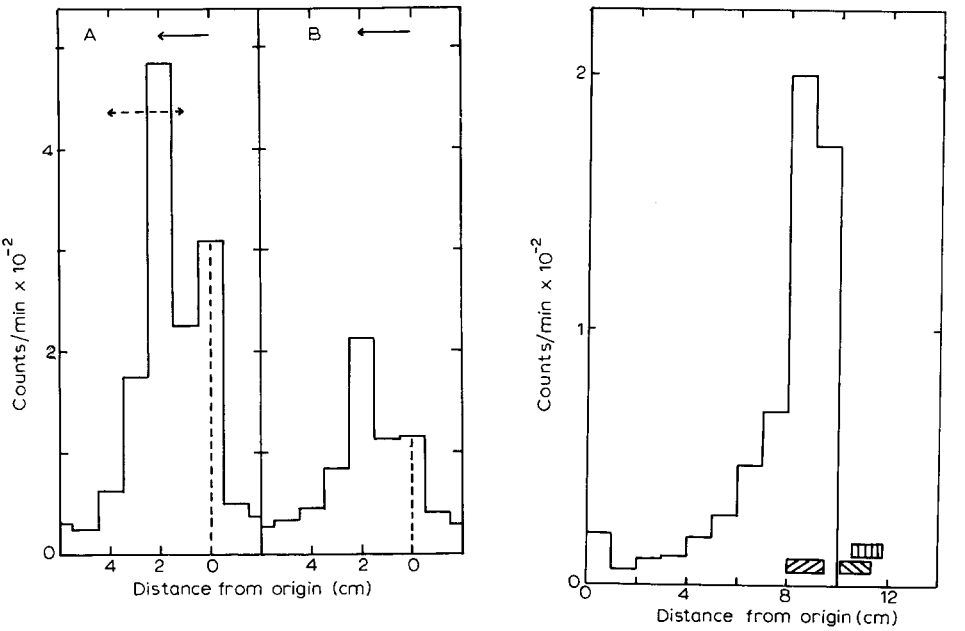


Fig. 5. Electrophoretic analysis of the [^{14}C]FDNB-labelled protein hydrolysate. A. Pronase hydrolysate of [^{14}C]FDNB labelled enzyme. B. Similar hydrolysate from the enzyme protected against FDNB action by the presence of acetyl-CoA. Equal aliquots of the two hydrolysates were applied to Whatman 3MM paper and electrophoresed for 2 h at pH 3.7. The areas of radioactivity were located by cutting the paper into pieces 1 cm wide and counting them in a Packard Tricarb Scintillation Spectrometer. The area in the sample lane between 1 and 4 cm from the starting line (←— — —→) was eluted and concentrated for chromatographic analysis. The arrows indicate the direction of the cathode.

Fig. 6. Chromatographic analysis of [^{14}C]DNP-amino acids. The material eluted from the electrophoretogram developed at pH 3.7 was analysed by ascending thin-layer chromatography. ε-DNP-lysine was included both as an internal and an external standard (▨). External standards of *O*-DNP-tyrosine (vertical lines) and *imid*-DNP-histidine (▧) were also included. Areas corresponding to DNP-amino acids were detected by their absorption at 260 mμ. The radioactive areas were located and counted as described in Fig. 5.

lysine. The fact that acetyl-CoA protected against the chemical modification by FDNB provided a suitable control for the labelling experiment.

The pronase hydrolysate of the [^{14}C]FDNB labelled enzyme was also subjected to electrophoresis at pH 6.5. The electrophoretogram revealed a major radioactive band which moved toward the cathode. Again the radioactivity of the corresponding band from an acetyl-CoA protected sample was decreased. The material was eluted, concentrated, and on chromatographic analysis was found to be homogeneous with respect to radioactivity which co-chromatographed with ε-DNP-lysine.

Acetyl-CoA protection against inactivation

In an effort to assign a role for the reactive lysine residue in the catalysis, various components of the pyruvate carboxylase reaction were tested for their ability to protect the enzyme against FDNB action. The results of the protection experiments are summarised in Table II. A similar experiment was carried out with TNBS and similar protection by acetyl-CoA was observed. The protection afforded by acetyl-CoA against

TABLE II

PROTECTION OF ENZYMIC ACTIVITY BY REACTION MIXTURE COMPONENTS

The enzyme was preincubated in a solution (final volume 0.4 ml) containing 40 μmoles of *N*-ethyl morpholine–HCl (pH 8.4), 0.15 unit of pyruvate carboxylase, 0.1 μmole of FDNB and various components of the enzyme assay mixture were added as indicated. After 20 min at 20°, aliquots were withdrawn and analysed for residual activity.

Addition	*Concn. (M)*	*% of original activity*
Control (no FDNB)	—	100
None	—	64
$MgATP^{2-}$	$6.25 \cdot 10^{-3}$	52
Sodium pyruvate	$1.0 \cdot 10^{-2}$	64
$NaHCO_3$	$1.0 \cdot 10^{-2}$	53
Acetyl-CoA	$2.5 \cdot 10^{-3}$	80

FDNB action was found to be concentration dependent (Fig. 7). From the data presented in Fig. 7 the pseudo first order rate constants for the inactivation process in the presence of varying concentrations of acetyl-CoA can be obtained. SCRUTTON AND

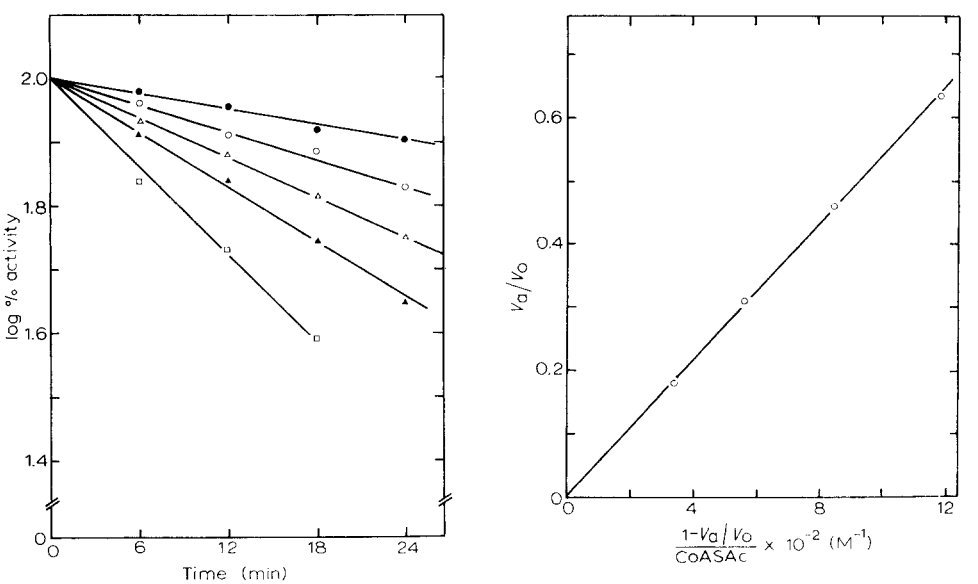


Fig. 7. Protection of pyruvate carboxylase by acetyl-CoA against FDNB inhibition. The enzyme was modified with FDNB, $2 \cdot 10^{-4}$ M, as described in Fig. 1 in the presence of varying concentrations of acetyl-CoA (final volume, 0.40 ml). The concentrations of acetyl-CoA used were: 0 (□—□), $3.1 \cdot 10^{-4}$ M (▲—▲), $6.25 \cdot 10^{-4}$ M (△—△), $1.25 \cdot 10^{-3}$ M (○--○), and $2.5 \cdot 10^{-3}$ M (●—●).

Fig. 8. Determination of the K_d (dissociation constant of the enzyme–acetyl-CoA complex). The reciprocal of the half-time ($t_{0.5}$) for protected (V_a) and unprotected (V_o) enzyme inhibited as described in Fig. 7 were plotted so that the ratio V_a/V_o was plotted as a function of $(1 - V_a/V_o)/$(acetyl-CoA).

UTTER[10] derived the equation

$$\frac{V_a}{V_o} = \frac{k_2}{k_1} + K_d\left(\frac{1 - V_a/V_o}{A}\right) \quad (6)$$

where V_a and V_o represent, respectively, the pseudo first order rate constants for inactivation in the presence and absence of A, the protecting agent, *i.e.* acetyl-CoA; k_1 and k_2 are the fractional order rate constants for inactivation of free enzyme (Eqn. 7) and the enzyme–acetyl-CoA complex (Eqn. 8), respectively; and K_d, the dissociation constant for EA (Eqn. 9).

$$E + nI \xrightarrow{k_1} EI_n \quad (7)$$

$$EA + nI \xrightarrow{k_2} EAI_n \quad (8)$$

$$E + A \overset{K_d}{\rightleftharpoons} EA \quad (9)$$

When the ratio of the pseudo first order constants for inactivation in the presence and absence of acetyl-CoA, *i.e.* V_a/V_o, is plotted against $(1 - V_a/V_o)/A$ the intercept represents k_2/k_1, *i.e.* the ratio of the fractional order rate constants for the reaction between free enzyme and enzyme–acetyl-CoA complex with inhibitor. The slope of the graph is K_d. Such a plot derived from the data obtained in Fig. 7 is presented in Fig. 8.

From the fact that the plot reveals a straight line which passes through the origin, it is concluded that FDNB cannot combine with the enzyme–acetyl-CoA complex, *i.e.* k_2 is very small compared with k_1. Using the same procedure SCRUTTON AND UTTER[10] have reported the complete protection of avian liver pyruvate carboxylase by ATP against inactivation by avidin, and EDWARDS AND KEECH[11] have demonstrated the complete protection of propionyl-CoA carboxylase by propionyl-CoA against inactivation by *N*-ethyl maleimide. The dissociation constant for the enzyme–acetyl-CoA complex was estimated to be $5.4 \cdot 10^{-4}$ M.

DISCUSSION

In the preceding section, evidence has been presented that the chemical modification of the ε-NH_2 group of a lysine residue in sheep kidney pyruvate carboxylase causes loss of enzymic activity. Amino groups in many enzymes are known to react with amino group reagents to yield a catalytically inactive product. For example, ribonuclease A[2,19], myosin A ATPase[1], phosphorylase[18], transaldolase[22], adenosine deaminase[3] and fructose 1,6-diphosphatase[4] are included in this category. Generally, however, no distinction can be made between the chemical modification of a reactive group at the active site of the enzyme and the production of an inactive enzyme by a reaction with groups elsewhere in the molecule which results in conformation changes. In this respect, WEBB[21] has distinguished between four possibilities: (a) the exposed reactive group may be at the active site and functional in the reaction mechanism; (b) the group may be at the active centre but non-functional in the reaction mechanism; (c) the group may be adjacent to the active site, or (d) the group may be removed from the active site and changes in enzyme activity result from conformation changes due to chemical modification. Because the demonstration of inhibition by amino group reagents can only indicate, at best, that one or more amino groups are sufficiently near the active site to interfere either directly or indirectly with the catalysis, evidence of

a functional role of the reactive groups must come from data other than in inhibition studies.

The protection experiments reported here show that the reactive lysine residue is involved in the reaction mechanism. The interaction of acetyl-CoA, but not pyruvate, bicarbonate or MgATP, with the enzyme, protects against inactivation by dinitrophenylation of the reactive lysine residue. The masking of the reactive lysine by acetyl-CoA can be used to suggest that the residue may be (a) involved in the binding of acetyl-CoA to the enzyme, or (b) involved in maintaining the correct ionic environment and conformation of the acetyl-CoA binding site. From our data, it is not possible to distinguish between these two possibilities.

The characterisation of the allosteric inhibitor sites of a number of enzymes, including aspartate transcarbamylase[22,23], fructose-1,6-diphosphatase[4] and glutamate dehydrogenase[13] has been possible by virtue of the fact that the destruction of these sites does not impair the catalytic activity of the enzyme but renders it non-susceptible to allosteric inhibition. However, difficulty is often encountered in the attempted characterisation of an allosteric activator site because its modification frequently destroys the enzymic activity. In the case of mammalian pyruvate carboxylases, the requirement for acetyl-CoA is absolute[5,24] and the failure of the enzyme to exhibit catalytic activity as a result of chemical modification can be interpreted as being due to destruction of either the catalytic and/or the allosteric site.

BARRITT, KEECH AND LING[25] and SCRUTTON AND UTTER[26] have demonstrated that at least 2 molecules of acetyl-CoA acting in a homotropic co-operative manner are involved in the activation of mammalian pyruvate carboxylases. Therefore, if the binding of each acetyl-CoA molecule to the enzyme is the same then we should expect to observe at least two reactive lysine residues. However, our data indicates that only 1 molecule of either FDNB or TNBS is required to inactivate the enzyme. A possible explanation for this apparent discrepancy is that only one allosteric site is exposed in the native enzyme and that modification of the lysine residue prevents the unmasking of the second site. Support for this suggestion comes from the fact that the kinetics of the modified enzyme, *i.e.*, after 50% of the enzymic activity had been inactivated, still yielded sigmoidal curves when velocity was plotted as a function of acetyl-CoA concentration. This indicated that the cooperativity with respect to acetyl-CoA was not changed by the chemical modification process.

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