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THE ESSENTIAL THIOL GROUP OF SHEEP KIDNEY MITOCHONDRIAL PHOSPHOENOLPYRUVATE CARBOXYKINASE

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SUMMARY

1. Evidence has been presented that the integrity of a cysteine residue in sheep kidney mitochondrial phosphoenolpyruvate carboxykinase (GTP:oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32) is essential for full expression of catalytic activity. Modification of the enzyme with sulphydryl group reagents such as *N*-ethylmaleimide, iodoacetamide, iodosobenzoic acid, 5,5′-dithiobis(2-nitrobenzoic acid), *p*-hydroxymercuribenzoic acid and *p*-hydroxymercuriphenylsulphonic acid all resulted in a loss of catalytic activity. Confirmation of the identity of the reactive residue as cysteine was achieved by characterising the radioactive derivative of the *N*-ethyl[1-^{14}C]maleimide-labelled enzyme.

2. *N*-ethylmaleimide modification of the enzyme was first order with respect to both time and inhibitor concentration. Analysis of the kinetic data showed that an average of one molecule of the inhibitor reacted with the enzyme causing inactivation. The pH dependence of *N*-ethylmaleimide modification shows an inflection at pH 7.3 corresponding to the pK_a of the reactive sulphydryl group.

3. Protection against *N*-ethylmaleimide modification was afforded by the nucleotide substrate and analogues in the preferred order ITP < IDP > IMP > inosine. The dissociation constant, K_D, for the enzyme–IDP complex was $8.04 \cdot 10^{-6}$ M $\pm$ 0.26.

4. Kinetic investigations of the modified enzyme showed that both $K_{m(app)}$ and v_{max} were decreased when phosphoenolpyruvate and IDP were the variable substrates while with HCO_3^- as the variable substrate mixed inhibition patterns were obtained depending on the degree of modification with decreases in v_{max}.

5. *N*-ethylmaleimide modification resulted in a completely inactive product. However, methylation of this sulphydryl residue yields a derivative which shows a small residual activity (about 5–10% of that of the native enzyme).

6. It is concluded that the function of the sulphydryl group of phosphoenolpyruvate carboxykinase is in some way associated with the catalytic process as distinct from binding.

INTRODUCTION

A detailed molecular description of enzymic catalysis demands the character-

isation of the role of the amino acid residues in the active site. Valuable information concerning the process may be obtained by the chemical modification of a limited number of reactive groups and evaluation of the consequent alteration in kinetic and/or physical properties of the enzyme. Such an approach would establish whether an essential group in the active site has been blocked or whether the modification of a group or groups elsewhere in the protein has caused non-specific changes leading to an inactive or desensitised enzyme.

The sulphydryl group of cysteine has all too readily been assigned a role in the enzymic processes because of its ready susceptibility to modifying reagents[1]. However, only a few cases have been documented where a sulphydryl group has been demonstrated to be intimately involved in the catalytic process forming an S-acyl intermediate: fatty acid synthetase[2], glyceraldehyde-3-phosphate dehydrogenase[3,4], glyoxalase[5], papain[6], ficin[7] and transglutaminase[8]. On the other hand, in the case of propionyl-CoA carboxylase[9], a sulphydryl group facilitates the binding of one of the substrates (propionyl-CoA) but plays no further role as v_{max} is unaffected by complete blocking of this group.

Phosphoenolpyruvate carboxykinase (GTP:oxaloacetate-carboxylase (transphosphorylating), EC 4.1.1.32) from chicken liver[10] and pig liver mitochondria[11] required the presence of reducing agents such as glutathione or cysteine to exhibit maximum activity while each was inactivated by sulphydryl group reagents such as *N*-ethylmaleimide and *p*-hydroxymercuribenzoate. Sheep kidney mitochondrial phosphoenolpyruvate carboxykinase shows a similar dependence for maximum activity on the presence of glutathione and a similar sensitivity to sulphydryl group reagents. This would suggest the dependence of catalytic activity on the presence of at least one free sulphydryl group. This report demonstrates that in sheep kidney mitochondrial phosphoenolpyruvate carboxykinase the integrity of a cysteine residue is, in fact, essential for enzymic activity and that its function is associated with some aspect of the catalytic process as distinct from binding.

MATERIALS AND METHODS

Phosphoenolpyruvate carboxykinase was prepared from freeze-dried sheep kidney cortical mitochondria by a method modified from that of CHANG AND LANE[11]. Enzymic activity was assayed using a $^{14}CO_2$ fixation method. Aliquots of the enzyme were incubated in reaction mixtures (total volume 0.5 ml) containing (in μmoles); imidazole (Cl^-) buffer, pH 6.5 (adjusted at 30°), 50; $NaHCO_3$ (approx. 10^5 counts/min per μmole), 5; phosphoenolpyruvate, 0.4; IDP, 0.5; $MnCl_2$, 1.5; reduced glutathione, 0.8; sodium glutamate, 5; pyridoxal phosphate, 0.02; aspartate transaminase, 17 μg in 1% bovine serum albumin solution. After incubation for 10 min at 30°, the reaction was stopped with 0.25 ml 10% (w/v) trichloroacetic acid. 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 1-inch squares of Whatman 3 MM 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[12].

Barium[^{14}C]carbonate was obtained from the Radiochemical Centre, Amersham, Great Britain and $^{14}CO_2$ distilled from perchloric acid into an equivalent amount

of NaOH. Reduced glutathione, IDP, *N*-ethylmaleimide, *p*-hydroxymercuribenzoate, *p*-hydroxymercuriphenylsulphonic acid and 5,5'-dithiobis(2-nitrobenzoic acid) were products of Sigma Chemical Co., St. Louis, Mo., U.S.A. Pronase was purchased from the California Corporation for Biochemical Research while iodoacetamide was a product of British Drug Houses Ltd., and iodosobenzoic acid of K and K Laboratories, Inc. U.S.A. Phosphoenolpyruvate (anhydrous monocyclohexylammonium salt was prepared by the method of CLARK AND KIRBY[13]. *N*-ethyl[1-^{14}C]maleimide was purchased from Schwarz Bioresearch, Inc., N.Y. Methyl iodide was a product of British Drug Houses, Ltd. and was distilled prior to use to remove traces of mercury.

Labelling of the enzyme with *N*-ethyl[1-^{14}C]maleimide was achieved by the addition of 0.75 mg of enzyme to a solution of 50 μmoles phosphate (pH 7.0) and 0.18 μmoles *N*-ethyl[1-^{14}C]maleimide (0.5 μC) at 20°. The reaction was stopped by the addition of 50 μmoles of reduced glutathione and the protein precipitated with 10% (w/v) trichloroacetic acid. The denatured protein was collected on an oxoid membrane held on a Millipore suction apparatus. Washing was effected with several aliquots of 10% (w/v) trichloroacetic acid containing $1 \cdot 10^{-3}$ M *N*-ethylmaleimide and finally 1% (w/v) acetic acid. The protein was dissolved in 0.1 M NH_4OH and the pH adjusted with formic acid to pH 7.5. The modified protein was hydrolysed with pronase for 72 h at 37° after which time hydrolysis had not gone to completion as shown by electrophoretic analysis. The hydrolysate was further hydrolysed by 6 M HCl at 106° for 16 h and 70 h. The HCl was removed under vacuum and the residue dissolved in a minimum quantity of glass-distilled water (0.15 ml) and used for electrophoretic analysis.

Electrophoresis was performed on Whatman 3 MM paper with pyridine–acetic acid–water (400:16:3600, v/v/v) buffer, pH 6.5, on a 'flat-bed' apparatus at 40 V/cm.

RESULTS

Inhibition using typical sulphydryl reagents

Sulphydryl-modifying reagents may broadly be divided into three groups (i) alkylating agents where the sulphydryl is covalently and irreversibly modified, (ii) mercaptide-forming agents which are reversible by incubation with excess of a second sulphydryl compound, *e.g.*, reduced glutathione, and (iii) oxidising agents where a disulphide bond is formed between two molecules of the protein or between the protein and the modifier. Inactivation of phosphoenolpyruvate carboxykinase was investigated with two reagents from each of the above three groups (Table I). The enzymic activity is seen to be susceptible to the reagents of each of these groups although iodoacetamide was much less active than the others.

N-ethylmaleimide has been shown by SMYTH, BLUMENFELD AND KONIGSBERG[14] and BREWER AND RHIEM[15] to react with both histidine and lysine but at a much reduced rate as compared with sulphydryl groups. Iodoacetamide will react slowly with histidine, tryptophan, the ε-NH_2 group of lysine, the -OH of tyrosine and the -SCH_3 of methionine[1] but no significant reaction occurs under the conditions for modification of the -SH groups. However, coupled with the almost complete specificity of the mercaptide and oxidising agents for free sulphydryl groups, we may conclude that the inactivation of phosphoenolpyruvate carboxykinase by these chemical modifiers indicates the presence of a cysteine residue in the enzyme whose integrity is essential for catalytic activity.

TABLE I

INHIBITION OF PHOSPHOENOLPYRUVATE CARBOXYKINASE BY SULPHYDRYL INHIBITORS

The enzyme was preincubated in a solution (final volume 0.1 ml) containing 5.0 μmoles phosphate (pH 7.0), 0.1 units phosphoenolpyruvate carboxykinase and sulphydryl inhibitor as indicated below. Incubation was at 20° for the indicated times when the reaction was halted by the addition of 0.025 ml 1% (w/v) bovine serum albumin containing 10^{-1} M glutathione, pH 7.0. Aliquots were withdrawn and analysed for residual activity. In the case of *p*-hydroxymercuribenzoate, incubation was at 30° in a solution containing 5.0 μmoles Tris, pH 8.0.

Reagent	*Concentration (M)*	*Time of incubation (min)*	*% Initial activity*
N-ethylmaleimide	$1 \cdot 10^{-4}$	10	22.8
Iodoacetamide	$5 \cdot 10^{-4}$	20	70.5
	$1 \cdot 10^{-4}$	30	77.4
Iodosobenzoate	$5 \cdot 10^{-4}$	20	29.0
	$1 \cdot 10^{-4}$	30	49.1
Dithiobis (2-nitrobenzoate)	$5 \cdot 10^{-4}$	2	70.7
	$1 \cdot 10^{-4}$	5	73.2
p-hydroxymercuriphenylsulphonate	$5 \cdot 10^{-4}$	2	13.2
	$1 \cdot 10^{-4}$	5	41.3
p-hydroxymercuribenzoate	$1 \cdot 10^{-4}$	2	5.3
	$2 \cdot 10^{-5}$	2	61.8

In the further experiments described here, the alkylating agent *N*-ethylmaleimide was used because modification was rapid and irreversible.

Order of inactivation with respect to time and N-ethylmaleimide concentration

Plots of log percentage of phosphoenolpyruvate carboxykinase activity as a function of time of *N*-ethylmaleimide inactivation were linear to the loss of 100% activity (Fig. 1) indicating that the inactivation process approximated first-order kinetics with respect to time at any fixed concentration of this inhibitor. The same data, when the rate of inactivation, expressed as the reciprocal of the half-time ($^1/t_{0.5}$), were replotted as a function of *N*-ethylmaleimide concentration (Fig. 2), showed that the inactivation process was pseudo first-order with respect to inhibitor concentration.

If one assumes the inactivation process to be,

$$E + nI \overset{k_1}{\rightarrow} EI_n \tag{1}$$

where E, I, EI_n, 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, the rate of the inactivation process, v, may be written as

$$v = k'[I]^n \tag{2}$$

where $k' = k_1[E]$ the pseudo first-order rate constant.

Expressing the rate of inactivation as the reciprocal of the half-time ($^1/t_{0.5}$), we obtain

$$\log (1/t_{0.5}) = \log k' + n \log[I] \tag{3}$$

such that when $\log (^1/t_{0.5})$ is plotted against $\log[I]$ a straight line is obtained of slope n,

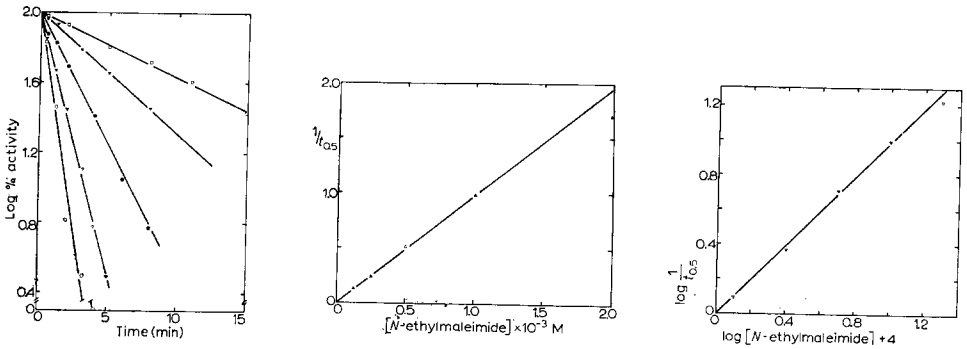


Fig. 1. The rate of inhibition of phosphoenolpyruvate carboxykinase with varying concentrations of *N*-ethylmaleimide. Enzyme was incubated at 20° in a solution (final volume 0.2 ml) containing 10.0 μmoles phosphate (pH 7.0), 0.3 units phosphoenolpyruvate carboxykinase and varying amounts of *N*-ethylmaleimide as indicated. Aliquots of 0.025 ml were diluted with 0.05 ml 1% (w/v) bovine serum albumin containing 10^{-1} M glutathione (pH 7.0) and analysed for residual activity as detailed in MATERIALS AND METHODS. Log_{10}% activity was plotted against time. The concentrations of *N*-ethylmaleimide were $2 \cdot 10^{-3}$ M (○—○); $1 \cdot 10^{-3}$ M (△—△); $5 \cdot 10^{-4}$ M (■—■); $2.5 \cdot 10^{-4}$ M (▲—▲); $1.25 \cdot 10^{-4}$ M (□—□).

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

Fig. 3. The data of Fig. 1 were replotted as $\log_{10}$ of the reciprocal of the half-time of inactivation against $\log_{10}$ concentration of *N*-ethylmaleimide.

i.e. the apparent number of molecules of inhibitor reacting per intact catalytic site to produce an inactive enzyme–inhibitor complex.

When the data of Fig. 1 are replotted in this manner (Fig. 3), the slope, $n = 1.0$, indicated that an average of one molecule of *N*-ethylmaleimide reacts with each catalytic site of the enzyme. Using a similar approach, LEVY, LEBER AND RYAN[16] have concluded that 3 moles of 2,4-dinitrophenol bind to one molecule of myosin causing inactivation. SCRUTTON AND UTTER[17] assumed at least two molecules of avidin were involved in the inactivation of avian liver pyruvate carboxylase based on a value of 1.4 for the slope of a similar plot. EDWARDS AND KEECH[9] have reported that one

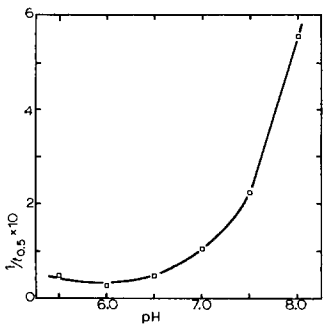


Fig. 4. Effect of pH on the rate of inactivation by *N*-ethylmaleimide. Conditions were as in Fig. 1 using $5 \cdot 10^{-2}$ M Tris phosphate buffer (pH 5.5–8.0) and 0.02 μmoles *N*-ethylmaleimide. The reciprocal of the half-time of inactivation was plotted against pH.

molecule of *N*-ethylmaleimide bound to pig heart propionyl-CoA carboxylase causing inactivation and KEECH AND FARRANT[18] showed the involvement of 1 molecule of 2,4-dinitrofluorobenzene in the inactivation of sheep kidney pyruvate carboxylase.

TABLE II

PROTECTION OF ENZYMIC ACTIVITY BY REACTION MIXTURE COMPONENTS

Conditions of preincubation were as in Table I with 0.01 μmoles *N*-ethylmaleimide and components of the assay mixture were added as indicated. A. Mn^{2+}: $2 \cdot 10^{-3}$ M; phosphoenolpyruvate: $2 \cdot 10^{-3}$ M; IDP: $2.5 \cdot 10^{-3}$ M; bicarbonate: $2 \cdot 10^{-2}$ M. Incubation, 10 min. B. Bicarbonate: $2 \cdot 10^{-3}$ M; all others were at $2 \cdot 10^{-4}$ M. Incubation, 19 min.

Addition	*% Original activity*
A. None	22.8
Mn^{2+}	37.2
Phosphoenolpyruvate	29.1
IDP	86.9
Bicarbonate	22.6
Phosphoenolpyruvate + Mn^{2+}	53.8
IDP + Mn^{2+}	83.0
Bicarbonate + Mn^{2+}	55.2
Phosphoenolpyruvate + IDP	86.0
B. None	4.8
ITP	33.5
IDP	80.8
IMP	24.6
Inosine	8.6
Inosine + phosphoenolpyruvate + Mn^{2+}	8.8
IMP + phosphoenolpyruvate + Mn^{2+}	27.0
ITP + phosphoenolpyruvate + Mn^{2+}	35.3
ITP + oxaloacetate + Mn^{2+}	39.7
Mn^{2+}	6.3
Phosphoenolpyruvate	6.2
Phosphoenolpyruvate + Mn^{2+}	5.1
Bicarbonate + Mn^{2+}	3.7

Effect of pH on the rate of inactivation by N-ethylmaleimide

EDWARDS AND KEECH[9] showed that *N*-ethylmaleimide inactivation of propionyl-CoA carboxylase was markedly dependent on the H^+ concentration. From a plot of rate of inactivation ($^1/t_{0.5}$) as a function of pH, an inflection point of 8.2 was obtained which corresponded to the pK_a of the group associated with propionyl-CoA binding as shown by a DIXON[19] plot when propionyl-CoA was varied. Further kinetic analysis of the modified enzyme confirmed the conclusion that the cysteine pK_a=8.2 which facilitated binding of propionyl-CoA was the group undergoing modification by *N*-ethylmaleimide.

The results of a similar investigation of the pH dependence of *N*-ethylmaleimide inactivation of phosphoenolpyruvate carboxykinase are shown in Fig. 4. The rate of inactivation remained constant as the pH increased to pH 7.0, but at higher pH values the rate of inactivation increased rapidly showing the ionised form of cysteine to be the reactive species. The intersection of the two linear sections of the graph at pH 7.3 is taken to be the pK_a of the reactive cysteine residue.

A pK_a value of 7.3 is low in comparison with the normal range of 8.5–9.2 for

cysteine in an electrostatically neutral environment, and would suggest the close proximity of a positively charged group[20]. Another example of a low pK_a value for the sulphydryl group was obtained by HOLLAWAY, MATHIAS AND RABIN[21] who found that the free thiol group of ficin reacted with iodoacetamide at a rate too fast to measure above pH 8.2. Moreover, RABIN AND WATTS[22] found the rate of iodoacetate reaction with creatine phosphokinase was independent of pH over the range 6.0–10.0 while the iodoacetate reaction with thiols shows a marked pH dependence over the range in which the thiol group ionises[23,24]. This evidence indicates complete ionisation of the thiol above pH 6.0, *i.e.* p$K_a \leqslant 6.0$, and would place the sulphydryl in a unique ionic environment.

IDP protection against N-ethylmaleimide inactivation

In an attempt to assign a role for the reactive cysteine residue in the enzymic process, various combinations of components of the phosphoenolpyruvate carboxykinase reaction were tested to assess their ability to protect the enzyme against *N* ethylmaleimide inactivation. Table II shows the results of these experiments. Almost complete protection was afforded by IDP or any combination of components containing IDP and the presence of the metal ion was not essential for IDP protection. Bicarbonate alone was the only component not to show any protection, but in the presence of Mn^{2+} some protection was evident. However, if the substrates were added at concentrations approximating their $K_{m\,(app)}$ value, there is an absolute specificity for the nucleotide with the preferred order ITP < IDP > IMP > inosine.

The protection afforded by IDP was found to be concentration dependent (Fig. 5). From this data, the pseudo first-order rate constants for the inactivation process in the presence of varying concentrations of IDP can be obtained. SCRUTTON AND UTTER[17] derived the equation

$$\frac{V_a}{V_o} = \frac{k_2}{k_1} + K_D \left(\frac{1 - V_a/V_o}{A}\right) \tag{4}$$

where V_a and V_o represent, respectively, the pseudo first-order rate constants for inactivation in the presence and absence of A, the substrate, *i.e.* IDP; k_1 and k_2 are the fractional-order rate constants for inactivation of free enzyme and enzyme–IDP complex and A is the concentration of protecting agent, *i.e.* IDP. When the ratios (V_a/V_o), of the pseudo first-order rate constants for inactivation in the presence and absence of IDP are plotted against

$$\left(\frac{1 - V_a/V_o}{A}\right)$$

the intercept represents k_2/k_1, the ratio of the fractional-order rate constants for the reaction between enzyme–IDP complex and free enzyme with the modifying agent. The slope of the line represents K_D, the dissociation constant. The data of Fig. 5 plotted in this manner are shown in Fig. 6.

The line has an intercept on the y-axis of 0.0165 ± 0.0118 such that the frequency with which this line would pass through the origin is small. It is thus concluded that IDP does not afford complete protection but that *N*-ethylmaleimide is able to combine with the enzyme–IDP binary complex although at a much reduced rate, *i.e.* k_2 is small compared with k_1. SCRUTTON AND UTTER[17], using this technique, have

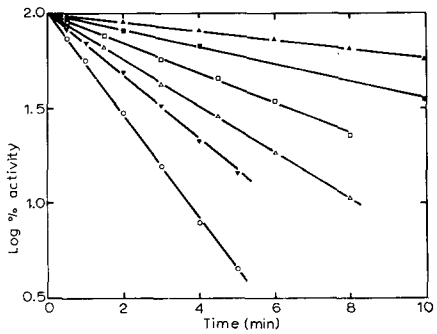


Fig. 5. Protection of *N*-ethylmaleimide inactivation by IDP. Conditions were as in Fig. 1 with 0.1 μmoles *N*-ethylmaleimide and varying concentrations of IDP were added as indicated. The reciprocal of the half-time of inactivation was plotted against time of incubation. The concentrations used were 0 (○—○); $1 \cdot 10^{-4}$ M (▲—▲); $4 \cdot 10^{-5}$ M (■—■); $2 \cdot 10^{-5}$ (□—□); $1 \cdot 10^{-5}$ M (△—△); $5 \cdot 10^{-6}$ M (▼—▼).

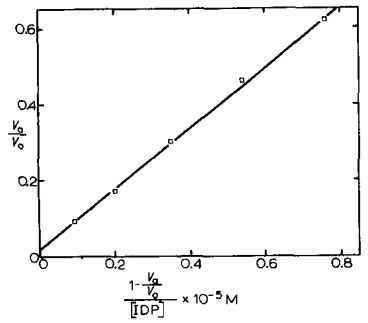


Fig. 6. Determination of the K_D (dissociation constant) of IDP. The $t_{0.5}$ of inactivation for enzyme in the presence (V_a) and absence (V_o) of the protecting agent, IDP, were obtained from Fig. 5 and the ratio V_a/V_o was plotted against $1 - V_a/V_o/[\text{IDP}]$.

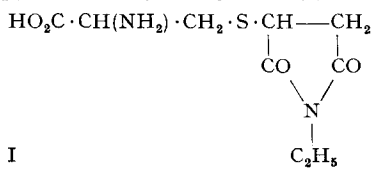
demonstrated the complete protection by ATP against avidin inactivation of avian liver pyruvate carboxylase, while in this laboratory, EDWARDS AND KEECH[9] showed complete protection of pig heart propionyl-CoA carboxylase by propionyl-CoA against *N*-ethylmaleimide and acetyl-CoA affords complete protection to sheep kidney pyruvate carboxylase against inactivation by 2,4-dinitrofluorobenzene[18].

The dissociation constant for the enzyme–IDP complex was calculated at $8.04 \cdot 10^{-6}$ M $\pm$ 0.26 which is significantly lower than the apparent K_m for this substrate: approx. $2 \cdot 10^{-4}$ M.

Identification of the N-ethyl[^{14}C]maleimide amino acid derivative from N-ethyl[^{14}C]-maleimide-labelled enzyme

A 16-h acid hydrolysate of *N*-ethyl[^{14}C]maleimide-labelled enzyme, following pronase digestion, was subjected to electrophoretic analysis at pH 6.5 (see MATERIALS AND METHODS). Analysis of the electrophoretogram revealed two major radioactive bands which moved in opposite directions. In a similar experiment where the enzyme was protected against alkylation by IDP ($5 \cdot 10^{-4}$ M) the radioactivity of both bands was reduced correspondingly by 40% (Fig. 7).

Analysis of the 70-h acid hydrolysate showed that the radioactive band which moved toward the anode increased while that band which moved slowly toward the cathode was diminished (Fig. 8A), indicative of the conversion of the one derivative to the other corresponding to the cleavage of the pyrrolidine ring of *S*-(1-ethyl-2,5-dioxopyrrolidin-3-yl)-L-cysteine(I)



I

and liberation of ethylamine to give *S*-(1,2-dicarboxyethyl)-L-cysteine(II),

$HO_2C \cdot CH(NH_2) \cdot CH_2 \cdot S \cdot CH(CO_2H) \cdot CH_2(CO_2H)$

II

the fast-moving component[14]. In all cases the fast- and slow-moving components were the only bands detected where radioactivity exceeded background.

The sample lane of the electrophoretograms between 1 cm and 3 cm from the origin toward the cathode and between 17 cm and 22 cm from the origin toward the anode was eluted with glass-distilled water, concentrated by freeze-drying and the two samples were subjected to electrophoretic analysis at pH 6.5. *S*-(1-ethyl-2,5-dioxopyrrolidin-3-yl)-L-cysteine (I) was included as an internal and external standard with the slow-moving component while *S*-(1,2 dicarboxyethyl)-L-cysteine (II) was included as an internal and external standard with the fast-moving component. The *N*-ethylmaleimide derivatives with L-histidine and L-lysine were included as external standards. Analysis of the electrophoretogram showed each sample was homogeneous with respect to radioactivity which co-electrophoresed with compound I in the former case and compound II in the latter case (Fig. 8B).

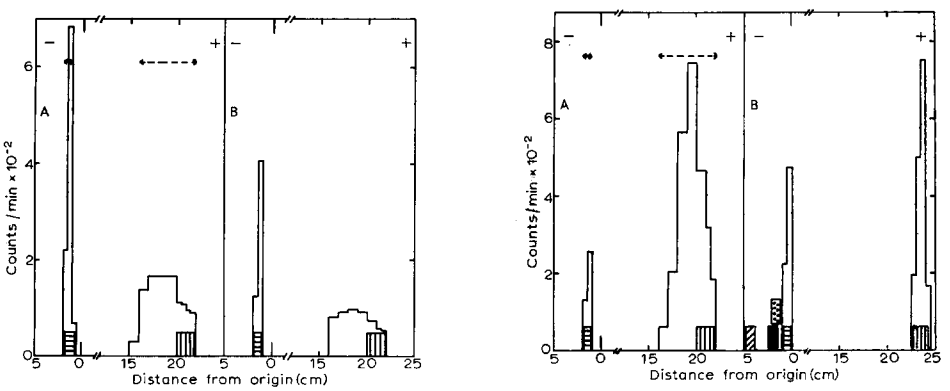


Fig. 7. Electrophoresis of 16-h acid digest of *N*-ethyl[^{14}C]maleimide labelled enzyme. The labelled enzyme after 16-h acid hydrolysis was subjected to electrophoresis at pH 6.5 as described in MATERIALS AND METHODS. This figure represents the radioactive profile obtained after 1-cm strips of the electrophoretogram were counted by scintillation counting. The standard compounds I and II were detected by ninhydrin. (A) absence of any protecting agent, and (B) 0.5 μmole IDP added during the labelling process. (horizontally hatched) *S*-ethyl-2,5-dioxopyrrolidin-3-yl)-L-cysteine (I); (vertically hatched) *S*-(1,2-dicarboxyethyl)-L-cysteine (II).

Fig. 8A. Electrophoresis of 70-h acid digest of *N*-ethyl[^{14}C]maleimide-enzyme. The labelled enzyme after 70-h acid hydrolysis was subjected to electrophoresis at pH 6.5 as described in MATERIALS AND METHODS. The electrophoretogram was analysed as described in Fig. 7. (horizontally hatched) *S*-(1-ethyl-2,5-dioxopyrrolidin-3-yl)-L-cysteine (I); (vertically hatched) *S*-(1,2-dicarboxyethyl)-L-cysteine (II).

Fig. 8B. Identification of the residue reactive towards *N*-ethylmaleimide. The sample lane of the electrophoretograms of Fig. 7A and B between 1 cm and 3 cm from the origin toward the cathode and between 17 cm and 22 cm from the origin toward the anode were eluted and electrophoresed as detailed in the text. *S*-(1-ethyl-2,5-dioxopyrrolidin-3-yl)-L-cysteine (I) was added as an internal and external standard with the former sample and *S*-(1,2-dicarboxyethyl)-L-cysteine (II) was included as an internal and external standard with the latter. (horizontally hatched) Compound (I); (vertically hatched) Compound (II); (dotted) *N*-ethylmaleimide-L-histidine; (diagonally hatched) α-*N*-ethylmaleimide-L-lysine; (black) β-*N*-ethylmaleimide-L-lysine.

Effect of chemical modification on the active site

Some insight into the role of a particular amino acid may be obtained by investigating the kinetics of an enzyme after modification of this group. Such an approach was used by KNOWLES[25] to show the involvement of methionine-192 in α-chymotrypsin with the binding of so-called "good" substrates or amides while with the "poor" substrates or esters, this group is essentially non-functional.

This approach has been used in the present investigation. LINEWEAVER–BURK[26] plots were constructed for two stages of modification (Figs. 9–11). A series of parallel

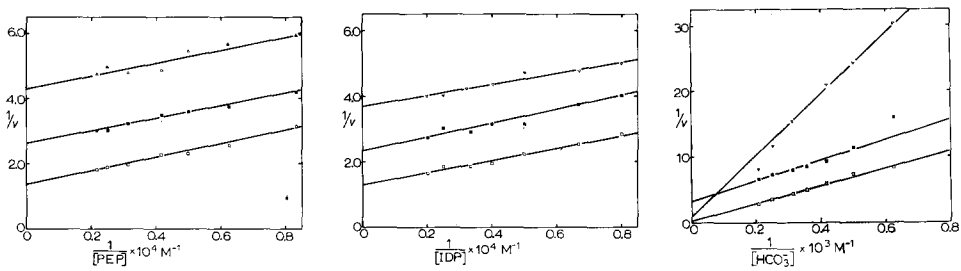


Fig. 9. Kinetic constants for phosphoenolpyruvate (PEP) in the chemically modified enzyme. Enzyme was incubated in a solution (final volume 0.8 ml) containing 40 μmoles phosphate buffer (pH 7.0), 0.20 μmoles *N*-ethylmaleimide and 1.2 units of enzyme. Aliquots of the enzyme (0.2 ml) were removed at 0, 3 and 7 min and diluted with 0.4 ml 1% bovine serum albumin containing 10^{-1} M reduced glutathione. The modified enzyme was then assayed using varying amounts of phosphoenolpyruvate. LINEWEAVER–BURK[26] plots of data after modification were for time 0 min (□—□); 3 min (■—■); and 7 min (△—△).

Fig. 10. Kinetic constants for IDP in the chemically modified enzyme. The enzyme was modified as described in Fig. 9. The enzyme was assayed using varying amounts of IDP and the LINEWEAVER–BURK[26] plots of data obtained after modification were for time 0 min (□—□); 3 min (■—■); 7 min (△—△).

Fig. 11. Kinetic constants for $NaHCO_3$ in the chemically modified enzyme. The enzyme was modified as described in Fig. 9. The enzyme was assayed using varying amounts of $NaHCO_3$ and the LINEWEAVER–BURK[26] plots of data obtained after modification were for time 0 min (□—□); 3 min (■—■); 7 min (△—△).

(or essentially so) lines was obtained with decreases in both $K_{m(app)}$ and v_{max} when phosphoenolpyruvate and IDP were the variable substrates. With HCO_3^- as the variable substrate, no clear-cut result was obtained, but rather a non-competitive type of inhibition pattern was approached for the higher degree of modification while at intermediate degrees of modification, a mixed pattern is obtained between non-competitive and uncompetitive inhibition patterns.

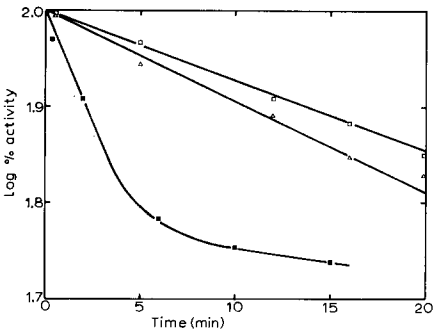
It is apparent that the loss of activity resulting from the alkylation of the sulphydryl group is not due to unsaturation of phosphoenolpyruvate, IDP or bicarbonate. In fact, excess IDP following modification causes a high degree of inhibition, possibly indicative of unimpaired binding of this substrate.

Methylation of the reaction sulphydryl group

The question which arises is whether the integrity of the sulphydryl group is essential for enzymic activity. Alkylation of the sulphydryl group using *N*-ethylmaleimide causes complete loss of activity. However, this could be the result of (a)

structural changes in the enzyme due to chemical modification, (b) steric considerations such that the approach of the sulphur atom toward the appropriate bond in the substrate is prevented, or (c) the electrostatic properties of the sulphur are sufficiently altered as to leave the residue catalytically inert. Therefore, methylation of the enzyme was attempted using methyliodide as the methylating agent. Modification by this reagent was inhibited by IDP suggesting that methylation was occurring at the same sulphydryl group as was alkylated by *N*-ethylmaleimide (Fig. 12).

N-ethylmaleimide will react only with the free sulphydryl group and not with its methylated derivative such that if *N*-ethylmaleimide were added after methyliodide modification, those enzyme molecules containing the unmodified sulphydryl group



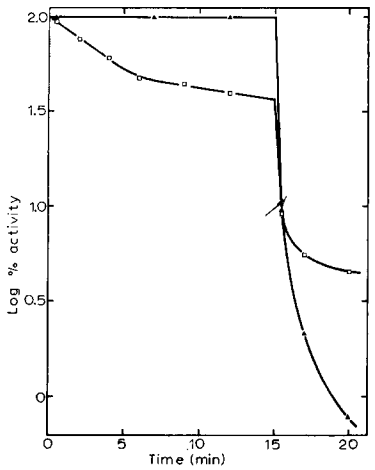


Fig. 12. Protection by IDP against modification by methyliodide. Enzyme was incubated at 30° in a solution (final volume 0.2 ml) containing 0.3 units enzyme, 10.0 μmoles Tris (pH 8.0) and 2.5 μmoles methyliodide. Varying amounts of IDP were added as indicated. Aliquots of 0.025 ml were diluted with 0.05 ml 1% (w/v) bovine serum albumin containing 10^{-1} M dithiothreitol (pH 7.0) and analysed for residual activity as detailed in MATERIALS AND METHODS. Log_{10}% activity was plotted against time. The concentration of IDP was 0 (■—■); $5 \cdot 10^{-4}$ M (□—□); $1 \cdot 10^{-4}$ M (△—△).

Fig. 13. Activity of enzyme modified by methyliodide. Conditions were as in Fig. 12 with 10 μmoles methyliodide. After 15 min incubation, 0.25 μmoles *N*-ethylmaleimide were added and the incubation was continued at 20°, aliquots being treated as before. Log_{10}% activity was plotted against time. (▲—▲), no methyliodide added; (□—□) in the presence of methyliodide.

would be inactivated by alkylation of this free, reactive sulphydryl group. Thus, if there were any residual activity remaining after such treatment, it must be due to those enzyme molecules containing the methylated sulphydryl residue. Fig. 13 presents the results of such an experiment and does indicate a low residual activity of the methylated enzyme.

DISCUSSION

Evidence has been presented that a sulphydryl group in phosphoenolpyruvate carboxykinase is alkylated by *N*-ethylmaleimide resulting in complete loss of activity. However, reaction with modifying reagents to give inactive enzymes does not preclude

the modification of sulphydryl groups remote from the active site so that through steric effects or through consequent structural changes a catalytically inactive product is formed. Thus, data other than from inhibition studies must be considered before the group is assigned a place in the active site and/or a role in the catalytic process.

A functional role for this specific cysteine in the active site is indicated, firstly, by the dependence of activity on the integrity of the sulphydryl group and, secondly, by the almost complete protection afforded by the substrate, IDP, against modification. However, as indicated in Fig. 6, this protection is never complete indicating that this group is, in fact, not strictly a binding group for IDP despite the almost complete protection with this substrate (Table II). Also, ITP the substrate for the decarboxylation of oxaloacetate, IMP, and inosine which all presumably bind at the same site as IDP, do not show the same degree of protection. This suggests an association of the sulphydryl group with the phosphate moiety of the nucleotide and may be indicative of a role for the sulphydryl group other than binding. This suggestion is strengthened by the absolute dependence on the intact sulphydryl group when *N*-ethylmaleimide is the modifying agent. One would expect that inhibition due to modification of a strictly binding group would not be complete since only the efficiency of binding would be impaired and such inhibition as is suffered would be reversed by excess substrate, *e.g.* the sulphydryl group of propionyl-CoA carboxylase[9] and the methionine of α-chymotrypsin[25]. However, with phosphoenolpyruvate carboxykinase, excess IDP, after modification, caused further marked substrate inhibition which is probably indicative of unimpaired binding of IDP.

Further, the kinetic evaluation shows a decrease in v_{max} when all substrates are varied. As $K_{m(app)}$ is a complex kinetic function, a decrease in v_{max} will also be reflected in a decrease in $K_{m(app)}$ if those kinetic constants being altered are significant in the K_m expression. This again strengthens the suggestion that the sulphydryl group has a catalytic function and is possibly associated with the phosphoryl transfer because of the parallel effects shown on the kinetic parameters associated with both phosphoenolpyruvate and IDP.

The fact that alkylation of the sulphydryl group by *N*-ethylmaleimide completely inactivates phosphoenolpyruvate carboxykinase while methylation of this same group results in some residual activity would indicate that the steric factors come into play with the more bulky *N*-ethylmaleimide residue, while with the less bulky methyl group the predominant effect is the decrease in the nucleophilicity of the sulphur atom.

Phosphoenolpyruvate carboxylase, phosphoenolpyruvate carboxytransphosphorylase and ribulose-diphosphate carboxylase, which, in common with phosphoenolpyruvate carboxykinase, are non-biotin carboxylating enzymes, all show a stimulation of catalytic activity in the presence of sulphydryl compounds[27–29] and a high sensitivity towards typical sulphydryl group reagents[30–33]. However, we have no information concerning the substrate associated with this residue although RACKER AND KRIMSKY[34] proposed an S-acyl intermediate between 3-phosphoglycerate and the sulphydryl in the action of ribulose diphosphate carboxylase. None of these enzymes use nucleotides as substrates while with the biotin-containing sulphydryl enzymes, propionyl-CoA carboxylase[9,35] and acetyl-CoA carboxylase[36], it is the acyl-CoA substrate that binds through the sulphydryl group. Thus, phosphoenolpyruvate carboxykinase appears to be a unique situation where the sulphydryl group appears to be a catalytic residue associated with the nucleotide substrate, IDP.

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