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SHEEP KIDNEY PHOSPHOENOLPYRUVATE CARBOXYLASE

FURTHER STUDIES ON THE SULPHYDRYL GROUPS USING DINITROFLUOROBENZENE AND *N*-ETHYLMALEIMIDE

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SUMMARY

Evidence is presented that two cysteine residues in sheep kidney mitochondrial phosphoenolpyruvate carboxylase (GTP:oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32) are essential for full enzymic activity. The identity of the reactive residues was established by isolating and identifying the radioactive derivative of the 1-fluoro-2,4-dinitro-[^{14}C]benzene-labelled enzyme.

One cysteinyl residue was protected by IDP against modification by either 1-fluoro-2,4-dinitrobenzene or 5,5'-dithiobis-(2-nitrobenzoic acid); the other was protected by Mn^{2+}.

Investigation of the chemically modified enzyme showed that the binding of only the nucleotide to the enzyme was altered. Furthermore, the nucleotide-independent and -dependent $^{14}CO_2$:oxaloacetate exchange reactions were inhibited by 1-fluoro-2,4-dinitrobenzene and *N*-ethylmaleimide modification to the same extent as the overall carboxylation reaction.

It is concluded that modification of the enzyme by 1-fluoro-2,4-dinitrobenzene and *N*-ethylmaleimide destroys some catalytic function associated with the carboxylation process *per se*, as well as the ability of the enzyme to bind the nucleotide substrate.

INTRODUCTION

Chemical modification procedures have been used extensively in attempts to identify the functional groups of enzymes. However, despite some limitations of the method such as lack of absolute specificity of most reagents and the difficulty in

Abbreviations: FDNB, 1-fluoro-2,4-dinitrobenzene; PEP, phosphoenolpyruvate; DNP-, dinitrophenyl.

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assigning an unambiguous role in the reaction mechanism for the functional group much valuable information may be obtained by using this approach[1,2].

In a previous report[3], evidence was presented which suggested that a sulphydryl residue of phosphoenolpyruvate (PEP) carboxylase (GTP:oxaloacetate carboxylyase (transphosphorylating), EC 4.1.1.32) was essential for full enzymic activity. The cysteinyl residue had an apparent catalytic function, it reacted with *N*-ethylmaleimide while IDP protected the enzyme against this chemical modification. In the present report, the inactivation of PEP carboxylase by reacting with 1-fluoro-2,4-dinitrobenzene (FDNB) is reported. FDNB was shown to react with two sulphydryl residues, one of which was protected by IDP, the other was protected by Mn^{2+}. In addition, the studies using *N*-ethylmaleimide were extended in an effort to assign a role in the catalytic process for the two reactive sulphydryl residues.

MATERIALS AND METHODS

The sources of all chemicals and enzymes were as previously described[3,4]. In addition, FDNB, 3,3'-dimethylglutaric acid and 5,5'-dithiobis-(2-nitrobenzoic acid) were products of Sigma. [^{14}C]FDNB was purchased from Schwarz Bioresearch and the standard dinitrophenyl- (DNP-) amino acids were purchased from Mann Research Laboratories except im-DNP-histidine which was prepared from *N*-acetylhistidine by the method of Margoliash[5].

2,4-Dinitrothiophenol was prepared by mixing equimolar amounts of 1-chloro-2,4-dinitrobenzene and Na_2S in acetone with stirring at room temperature. The acetone was removed using a rotary evaporator and the residue was dissolved in 1 M NH_4OH containing 10 mM 2-mercaptoethanol. This solution was filtered, the 2,4-dinitrothiophenol was precipitated by acidifying with formic acid and the precipitate was recovered by filtering. The 2,4-dinitrothiophenol was purified by dissolving the precipitate in 0.1 M NH_4OH containing 10 mM 2-mercaptoethanol and acidifying with formic acid. The precipitate was recovered by filtering, washed with cold 0.1 M formic acid and dried *in vacuo* for 72 h.

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

The enzyme was labelled by incubating 10 mg of enzyme with 100 μmoles of *N*-ethylmorpholine (Cl^-) (pH 7.5), and 0.5 μmole of [^{14}C]FDNB for 10 min at 20 °C (total volume 2 ml). The reaction was stopped by adding 400 μmoles of lysine (pH 7.5) and the protein was precipitated with 10% (w/v) trichloroacetic acid. The denatured protein was collected on a Whatman Glass Fibre Filter (GF/C) held in a Millipore suction apparatus. The protein precipitate was washed with several aliquots of 10% (w/v) trichloroacetic acid containing 10^{-3} M ε-DNP-lysine and finally with 1% (w/v) acetic acid. The modified protein was hydrolysed for 32 h at 106 °C with 6 M HCl. The HCl was removed under vacuum and the residue was dissolved in 1 ml of glass-distilled water.

Identification of the [^{14}C]DNP-amino acids was achieved by descending paper chromatography in the solvent systems, 1-butanol–acetic acid–water (4:1:1, v/v/v) or 1-butanol–water–ammonia (87:12:1, v/v/v). The papers were dried at 60 °C for 10 min and the position of the radioactivity was located in 1-cm strips of the sample lane of the chromatograph. The standard DNP-amino acids and their decomposition products,

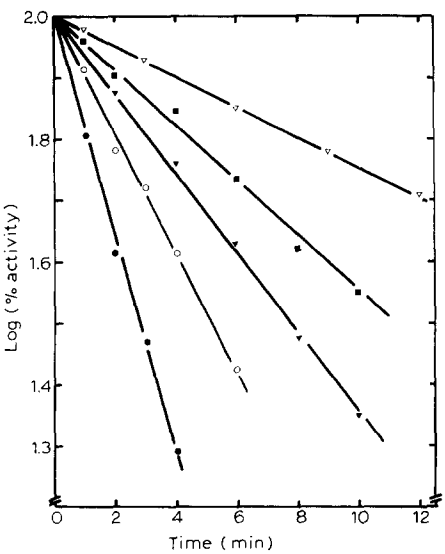
2,4-dinitroaniline, 2,4-dinitrophenol and 2,4-dinitrothiophenol, were detected by their absorption of ultraviolet light. *S*-DNP-cysteine and 2,4-dinitrothiophenol oxidized slowly in air and it was necessary to add 2-mercaptoethanol (10 mM) to both the standard solutions and the chromatographic solvents.

RESULTS

Preliminary studies showed that PEP carboxylase was inactivated by FDNB and 2,4,6-trinitrobenzene sulphonic acid. In the experiments reported here FDNB was used routinely because this reagent was available in a radioactive form. However, analysis of the kinetics of the inactivation process by either reagent, indicated that the reaction with the enzyme was the same in both cases.

Order of inactivation with respect to time and FDNB concentration

Plots of log percentage of PEP carboxylase activity as a function of time of inactivation were linear to the loss of at least 80% of the initial activity (Fig. 1), indi-



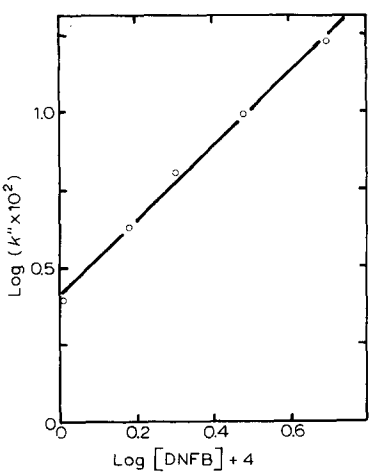


Fig. 1. The rate of inactivation of PEP carboxylase with varying FDNB concentrations. Enzyme was incubated at 25 °C in a solution (0.4 ml) containing 20 μmoles of *N*-ethylmorpholine (Cl^-) (pH 7.5), 0.4 unit of enzyme and FDNB as indicated. Aliquots of 0.05 ml were diluted with 0.325 ml of 1% (w/v) bovine serum albumin containing 10^{-1} M lysine and 10^{-4} M GSH (pH 7.5), and analysed for residual activity as described in Materials and Methods. $\log_{10}$ (% activity) was plotted against time. The FDNB concentrations were: ●—●, 0.5 mM; ○—○, 0.3 mM; ▼—▼, 0.2 mM; ■—■, 0.15 mM; ▽—▽, 0.1 mM.

Fig. 2. The data of Fig. 1 were replotted as $\log_{10} k''$ against log $_{10}$FDNB concentration.

cating that the inactivation process approximated first-order kinetics with respect to time at fixed concentrations of inhibitor. These data were replotted according to the equation[3,6]:

$$\log_{10} k'' = \log_{10} k' + n \cdot \log_{10} I \qquad (1)$$

where k'' is the rate of the inactivation process expressed as the slope of the primary plot (Fig. 1), k' is the pseudo first-order rate constant of the inactivation process, n is the number of FDNB molecules reacting per active site and I is the FDNB concentration. This plot (Fig. 2) gave a straight line with a slope of 1.25 indicating that more than one molecule of FDNB was reacting per catalytic unit of the enzyme to cause inactivation, *i.e.* more than one amino acid residue was being modified by FDNB. A similar experiment using trinitrobenzene sulphonic acid gave a slope of 1.2 for the log plot.

Protection by substrates against FDNB inactivation

The ability of various combinations of substrates to protect the enzyme against FDNB inactivation was tested (Table I). It can be seen that both IDP, which protects against *N*-ethylmaleimide inactivation[3], and Mn^{2+}, which has no influence on *N*-ethylmaleimide inactivation, separately protect the active site against chemical modification. However, the protection observed when Mn^{2+} was present was considerably greater than that afforded by IDP suggesting that a group different from that susceptible to *N*-ethylmaleimide treatment was being modified preferentially by FDNB. Mg^{2+} was completely ineffective in protecting against FDNB modification despite the fact that Mg^{2+} does activate the enzyme at the pH used here (pH 7.5)[4].

TABLE I

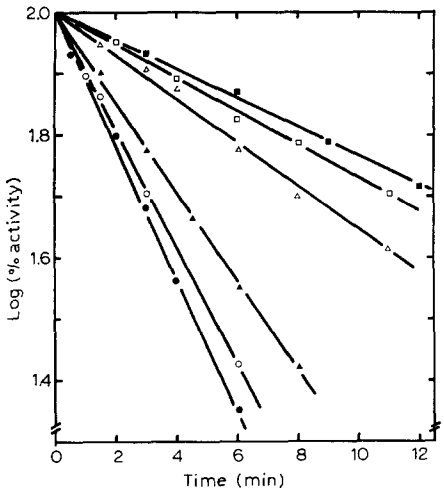
PROTECTION OF ENZYMIC ACTIVITY BY REACTION MIXTURE COMPONENTS

The enzyme was incubated for 6 min at 20 °C in a solution (final volume 0.2 ml) containing 10.0 μmoles *N*-ethylmorpholine (Cl^-) (pH 7.5), 0.2 unit enzyme, 0.06 μmole FDNB and substrates, as indicated, at $2 \cdot 10^{-4}$ M except $NaHCO_3$ which was at $2 \cdot 10^{-3}$ M. Aliquots of 0.025 ml were withdrawn, diluted with 0.05 ml of 1% (w/v) bovine serum albumin containing 10^{-1} M lysine, 10^{-4} M GSH (pH 7.5) and analysed for residual activity.

Additions	*% original activity*
None	34.5
Mn^{2+}	83.7
PEP	35.8
IDP	56.6
$NaHCO_3$	28.0
Mn^{2+} + PEP	82.0
Mn^{2+} + IDP	83.2
Mn^{2+} + $NaHCO_3$	79.0
Mg^{2+}	34.5
Mg^{2+} + PEP	32.8
Mg^{2+} + IDP	61.0
Mg^{2+} + $NaHCO_3$	33.0
Mn^{2+} + Mg^{2+}	72.8

The protection afforded by Mn^{2+} was concentration dependent (Fig. 3). These data have been plotted according to the method of Scrutton and Utter[7] using the equation:

$$\frac{V_a}{V_0} = \frac{k_2}{k_1} + K_D \frac{1 - V_a/V_0}{A} \qquad (2)$$



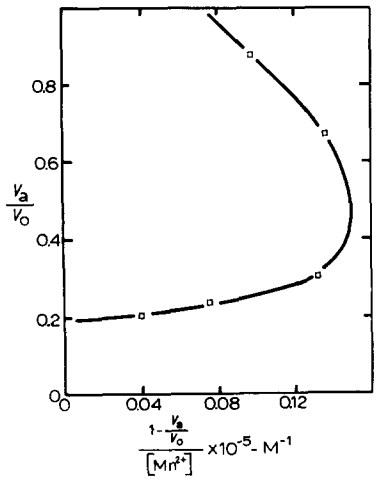


Fig. 3. Protection of FDNB inactivation by Mn^{2+}. Conditions were as detailed for Fig. 1 with 0.12 μmole FDNB and varying Mn^{2+} levels as indicated. log (% activity) was plotted against time. Mn^{2+} concentrations were: ●—●, 0.0 mM; ○—○, 0.0125 mM; ▲—▲, 0.025 mM; △—△, 0.05 mM; □—□, 0.1 mM; ■—■, 0.2 mM.

Fig. 4. The rate of FDNB inactivation in the presence (V_a) and absence (V_0) of Mn^{2+} was obtained from Fig. 3 and the ratio V_a/V_0 was plotted against $(1 - V_a/V_0)/Mn^{2+}$.

where V_a and V_0 represent, respectively, the pseudo first-order rate constants for inactivation (expressed as the slopes of the primary plots) in the presence and absence of A, the protecting agent (Mn^{2+}), k_1 and k_2 are the fractional-order rate constants for the inactivation of free enzyme and enzyme Mn^{2+} complex, respectively, and A is the concentration of Mn^{2+}. However, non-linear plots were obtained (Fig. 4). This was also the case when IDP was the protecting agent. Since non-linearity would be expected if modification occurred at a residue other than the one protected by the ligand under investigation, this provides further evidence that more than one residue was being modified by FDNB. A similar result for the secondary plot was obtained for Mn^{2+} protection against trinitrobenzene sulphonic acid inactivation.

Number of sulphydryl residues interacting with Mn^{2+} and IDP

Since the previous data indicated that more than one cysteinyl residue was being modified by FDNB, the number of sulphydryl residues interacting with Mn^{2+} and IDP was estimated using Ellman's reagent[8]. The reaction with 5,5'-dithiobis(2-nitrobenzoic acid) was followed spectrophotometrically at 412 nm at pH 7.0 and 20 °C and, under the conditions employed, and based on a molecular weight of 71 000 (ref. 4), a reaction with one residue per mole of enzyme would cause an increase of 0.14 absorbance units. There was an initial rapid increase in absorbance, corresponding to two sulphydryl groups per mole of enzyme, followed after 5 min, by a further increase in absorbance which could not be quantitated since the increase was caused largely by turbidity as the protein precipitated. In the presence of 2 mM Mn^{2+} or 2.5 mM IDP, there was a decrease in the rate of reaction with Ellman's reagent, while in the presence of both 2.5 mM IDP and 4 mM Mn^{2+}, the protective effect was additive corresponding to the protection of both of the thiol groups initially modified (Fig. 5).

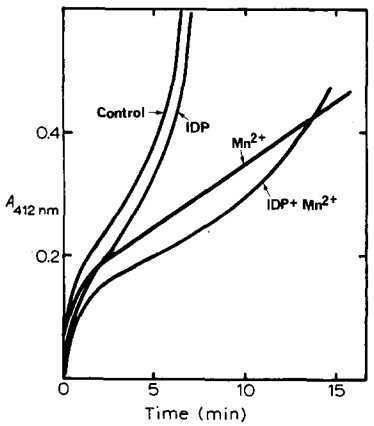


Fig. 5. The number of sulphydryl groups interacting with IDP and Mn^{2+}. Enzyme ($1.03 \cdot 10^{-5}$ M) was incubated at 20 °C with $1 \cdot 10^{-4}$ M, 5,5′-dithiobis-(2-nitrobenzoic acid) in $4 \cdot 10^{-2}$ M *N*-ethylmorpholine (Cl^-) (pH 7.0). The reaction was followed spectrophotometrically at 412 nm. Under these conditions, an absorbance change of 0.14 unit corresponds to 1 sulphydryl residue per mole of enzyme. Final concentrations of IDP and Mn^{2+} were 2.5 and 2.0 mM, respectively, except where both IDP and Mn^{2+} were present and the latter then was 4.0 mM.

Mn^{2+} also showed the additional effect of preventing the precipitation of the enzyme following modification.

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

A 32-h acid hydrolysate of the [^{14}C]FDNB-labelled enzyme was subjected to chromatographic analysis (see Materials and Methods). In the acid solvent, a major radioactive band corresponding to *S*-DNP-cysteine was revealed along with a slower

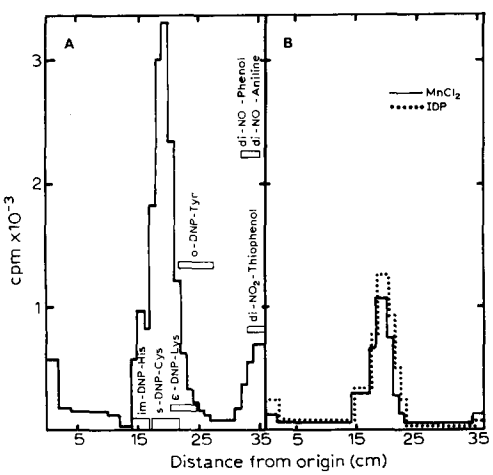


Fig. 6. Chromatography of a 32 h acid digest of [^{14}C]FDNB-labelled enzyme. The labelled enzyme after 32 h acid hydrolysis was subjected to chromatography in 1-butanol–acetic acid–water (4:1:1, v/v/v) on Whatman No. 4 paper for 4.5 h and analysed for radioactivity as described in Materials and Methods. The standard compounds were determined as dark spots under ultraviolet light. A; absence of any protecting agent, and B; 0.5 mM $MnCl_2$ (———) or 0.5 mM IDP (· · · · · ·) added during the labelling process.

moving compound corresponding to im-DNP-histidine (Fig. 6A). A similar result was also obtained using the alkaline solvent. In similar experiments, where the enzyme was protected by 0.5 mM Mn^{2+} or 0.5 mM IDP, the amount of radioactivity incorporated into the enzyme was uniformly reduced by 45 and 40%, respectively (Fig. 6B).

Elution of the sample lane of the chromatogram (Fig. 6A) 14–22 cm from the origin with glass-distilled water and rechromatography in the same solvent showed that the DNP-derivative had apparently undergone decomposition as indicated by the appearance of a radioactive band moving almost with the solvent front. DNP-amino acids are photosensitive and decompose to a substituted phenol or aniline. Chromatography in both acid and alkaline solvents confirmed that the decomposition product in this case was 2,4-dinitrothiophenol, the decomposition product of *S*-DNP-cysteine (Fig. 7). Thus, cysteine residues were labelled preferentially by FDNB but with a minor reaction at a histidine residue.

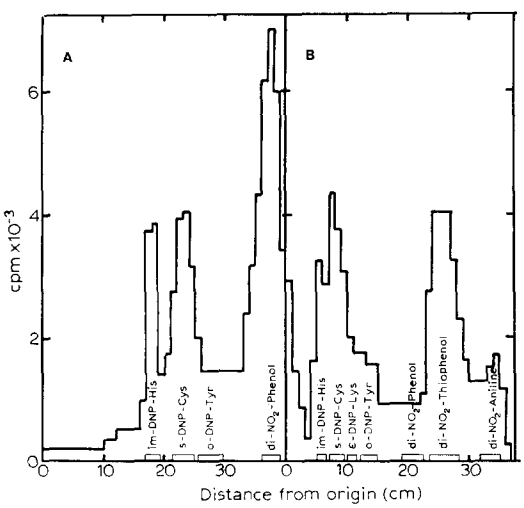


Fig. 7. Identification of the residue reactive towards FDNB. The sample lane of the chromatogram of Fig. 6A between 14 and 22 cm from the origin was eluted with glass-distilled water and rechromatographed on Whatman No. 4 paper for 7.5 h with the two solvents: 1-butanol–acetic acid–water (4:1:1, v/v/v) (A) and 1-butanol–water–conc. ammonia (87:12:1, v/v/v) (B). The chromatograms were processed as in Fig. 6.

Effect of chemical modification on the active site

In the investigation on the *N*-ethylmaleimide-susceptible sulphydryl group[3], analysis of the kinetic properties of the enzyme after modification was used to help determine the role of the reactive group. This approach was made possible by the fact that only one cysteinyl group was being modified. However, in the case of FDNB modification, such an approach could not be applied because it would be impossible to differentiate between the effects produced by modification of the two groups.

An alternative approach to this question was attempted whereby the rate of inactivation by FDNB was assessed by assaying the residual activity in six different ways, *viz.* carboxylation of PEP, and the $^{14}CO_2$:oxaloacetate exchange reaction in the presence of (i) Mn^{2+}, (ii) ITP and Mn^{2+}, (iii) IDP and Mn^{2+}, (iv) ITP and Mg^{2+}, (v) IDP and Mg^{2+}. It was hoped that this approach would differentiate between effects

on the carboxylation and phosphoryl transfer processes as well as detecting any specificity changes with respect to metal ion and nucleotide. Table II shows, however, that the rate of inactivation was the same in all cases. A similar result was obtained with *N*-ethylmaleimide inactivation.

TABLE II

EFFECT OF FDNB MODIFICATION OF THE ACTIVE SITE

A. The enzyme was incubated in a solution (final volume 0.4 ml) containing 20 μmoles *N*-ethylmorpholine (Cl^-) (pH 7.5), 0.35 unit enzyme and 0.05 μmole FDNB. Incubation was at 25 °C. At various times, 0.05-ml aliquots were diluted with 0.1 ml of 1% (w/v) bovine serum albumin containing 10^{-1} M lysine and 10^{-4} M GSH (pH 7.5) and analysed for residual activity by the methods listed above. Half-times of inactivation were obtained from semi-log plots of residual activity against time. The carboxylation assay was standard[3]. The exchange assay (total volume 0.5 ml) contained (in μmoles): imidazole (Cl^-) (pH 6.5) 50.0; Mn^{2+} or Mg^{2+}, 2.0; nucleotide (if present), 1.0; oxaloacetate, 1.0; $NaH^{14}CO_3$, 20.0; GSH, 0.8 and incubation was for 4 min. Processing of all assays was as previously described[3,4]. B. Conditions were the same as above except that 0.04 μmole of *N*-ethylmaleimide were used in the preincubation and aliquots of this solution were diluted with 0.1 ml of 1% (w/v) bovine serum albumin containing 10^{-1} M glutathione (pH 7.5).

Method of assay	*Half-time of inactivation (min)*	
	A	*B*
Carboxylation	3.1	5.1
CO_2:oxaloacetate exchange (Mn^{2+}/no nucleotide)	3.1	5.04
CO_2:oxaloacetate exchange (Mn^{2+}/ITP)	3.05	5.22
CO_2:oxaloacetate exchange (Mn^{2+}/IDP)	3.0	5.15
CO_2:oxalocaetate exchange (Mg^{2+}/ITP)	3.15	5.17
CO_2:oxaloacetate exchange (Mg^{2+}/IDP)	3.15	5.15

A third approach to this question is provided by proton relaxation rate studies similar to that used by O'Sullivan and Cohn[9] with creatine kinase (EC 2.7.3.2). These workers established that the quantitative reaction of two sulphydryl groups with iodoacetic acid inactivated the enzyme while the presence of nucleotides decreased the modification rate. However, the binding of nucleotide was in no way affected by this modification since the inactivated enzyme bound $Mn \cdot ADP^-$ with the same dissociation constant and the same proton relaxation rate enhancement factor as the native enzyme, *i.e.* the nucleotide afforded protection against inactivation by inducing a conformational change on binding to the enzyme.

Modification of PEP carboxylase by *N*-ethylmaleimide and FDNB was allowed to proceed at pH 7.5 for two half-times at which time the enhancement of proton relaxation rate (ε^*) was assessed[9,10]. With FDNB, Mn^{2+} was present during the inactivation process so that the same cysteinyl residue was being modified by either reagent, *viz.*, that group which is protected by IDP. It was found that Mn^{2+} and PEP binding was apparently unimpaired (*i.e.* no change in ε^* provided the incubation period was not excessive). However, the decrease in ε^* following nucleotide binding was abolished after reaction with either FDNB or *N*-ethylmaleimide indicating that either the nucleotide could no longer bind to the modified enzyme or a conformational change induced by the nucleotide and responsible for the observed change in ε^* could no longer occur on nucleotide binding. Using the method of Colowick and Womack[11], it was found that the ability of PEP carboxylase to bind nucleotide at pH 7.5 was abolished after *N*-

ethylmaleimide inactivation. Thus, the modification of one of the two reactive cysteinyl residues in some way alters the nucleotide binding site.

DISCUSSION

Evidence is presented here and in a previous report[3] that sulphydryl groups of PEP carboxylase are modified by reaction with FDNB and *N*-ethylmaleimide with concurrent loss of catalytic activity. Analysis of the kinetics of the inactivation process by these two reagents indicated that, although only one cysteinyl residue reacted with *N*-ethylmaleimide[3], more than one residue reacted with DNFB (Figs 2 and 4). The presence of two reactive sulphydryl groups was confirmed by titration of the enzyme with 5,5′-dithiobis-(2-nitro-benzoic acid) (Fig. 5).

Inactivation by trinitrobenzene sulphonic acid showed essentially similar characteristics as did FDNB (a value of 1.2 for n (Eqn 1) compared with 1.25 with using FDNB and a similar response to varying pH and Mn^{2+} and IDP protection). Since both trinitrobenzene sulphonic acid and FDNB are known to react with both cysteinyl and lysyl residues provided they are suitably activated and accessible[2,12–14], it is reasonable to assume that both trinitrobenzene sulphonic acid and FDNB modified the same sulphydryl groups.

The high reactivity of the sulphydryl groups in enzymes has led some investigators to postulate their participation at the active sites of many enzymes[15]. In very few instances, however, has it been shown that the sulphydryl groups form part of the active site and become involved in the catalytic sequence. The sulphydryl group has been implicated in the formation of S-acyl intermediates in some enzymes[16–23], it is known to be involved in the binding of substrate in at least one enzyme[24] while modification of this group is known to cause the loss of cooperative interactions in at least two other enzymes[25–27]. In the case of PEP carboxylase, although two sulphydryl groups are known to be essential for enzymic activity their precise role is not clearly defined.

Since the ability of the enzyme to bind the nucleotide is destroyed following inactivation by *N*-ethylmaleimide and FDNB, it is significant that the nucleotide-independent $^{14}CO_2$:oxaloacetate exchange reaction is inhibited to the same degree as the nucleotide-dependent activity (Table II), *i.e.* some catalytic function associated with the carboxylation process is destroyed. Therefore, since *N*-ethylmaleimide appears to modify only one sulphydryl group[3] this group could not be solely a nucleotide binding group. This result is in agreement with our previous conclusions[3].

O'Sullivan and Cohn[9] have presented evidence that the nucleotide protects against iodoacetate inactivation of creatine kinase by inducing a conformational change upon binding to the enzyme. Although the nucleotide substrate of PEP carboxylase does appear to activate the Mn^{2+}-dependent $^{14}CO_2$:oxaloacetate exchange reaction by inducing a conformational change[4], there is no evidence to indicate that protection against modification by *N*-ethylmaleimide and FDNB occurs by this means since the protection afforded by IDP was in no way altered by the presence of other substrates. On the other hand, structural changes in the chemically modified protein may contribute in some way to the loss of catalytic activity following chemical modification since attempts to recover enzymic activity following contact with the so-called "reversible" inhibitors, such as mercurials and 5,5′-dithiobis-(2-nitrobenzoic

acid) were unsuccessful. However, any such changes must not encompass the whole molecule since, apart from the nucleotide binding site, the binding sites for the other substrates were unaffected.

Since IDP afforded partial protection against both FDNB and *N*-ethylmaleimide modification, it would appear that both reagents react with the same cysteinyl group. This group must be in a reasonably accessible position since IDP protects against inactivation by several sulphydryl reagents of vastly different properties[3]. The sulphydryl group protected by Mn^{2+} could be in a more hydrophobic environment since its modification by the more hydrophilic reagent does not seem to occur.

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