The Reaction of Rabbit Muscle Creatine Kinase with Diethyl Pyrocarbonate

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The reaction of rabbit muscle creatine kinase with diethyl pyrocarbonate was studied. It was found that up to five of the sixteen histidine groups per enzyme subunit could be modified, and under the conditions employed, there was no evidence for formation of the disubstituted derivative of histidine. Evidence was obtained for small but significant amounts of modification of lysine and cysteine groups; tyrosine groups were not modified. Modification of the enzyme led to inactivation; this could be protected against by inclusion of substrates or, more effectively, by inclusion of the combination MgADP plus creatine plus nitrate, which is thought to produce a 'transition-stage-analogue' complex. Analysis of data on the rates of inactivation and the stoichiometry of modification suggested that there was one essential histidine group per enzyme subunit, modification of which led to inactivation.

Although extensive chemical-modification studies of creatine kinase (ATP-creatine *N*-phosphotransferase; EC 2.7.3.2) have been carried out (Watts, 1973; Bickerstaff & Price, 1978a), relatively little is known about the nature of groups at the active site of the enzyme. Studies of the hydrolysis of phosphocreatine have indicated the importance of a proton-donating/accepting group in the enzyme-catalysed reaction (Allen & Haake, 1976). In view of the fact that the pH-activity curve for the enzyme-catalysed reaction is a typical titration curve with a pK_a of about 6.5 (Noda *et al.*, 1960; Watts, 1973), it is tempting to speculate that a histidine group on the enzyme may be this proton-donating/accepting species.

Pradel & Kassab (1968) studied the modification of creatine kinase by diethyl pyrocarbonate, and concluded that reaction of a single histidine group per enzyme subunit led to inactivation. However, they did not investigate in detail either the protection afforded by substrates, or the specificity of the modification reaction. It is now known that diethyl pyrocarbonate can react with a variety of nucleophilic side chains in proteins (Miles, 1977). In the present paper, we report the results of a more detailed investigation of the reaction between creatine kinase and diethyl pyrocarbonate, and present evidence to support the involvement of a single histidine group in the mechanism of action of the enzyme.

Abbreviation used: Nbs₂, 5,5'-dithiobis-(2-nitrobenzoic acid).

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Materials and Methods

Creatine kinase was isolated from rabbit skeletal muscle and routinely assayed as described previously (Bickerstaff & Price, 1978b). Reversible protection of the reactive cysteine group in the enzyme was achieved by using potassium tetrathionate (Kassab *et al.*, 1968). Potassium tetrathionate was prepared by the method of Trudinger (1961). It should be noted that assays performed on the enzyme with intact cysteine groups were carried out without prior incubation with dithiothreitol, whereas those on the thiol-protected enzyme were carried out after incubation with this reagent.

Hydroxylamine hydrochloride, *N*-acetyl-L-histidine, Nbs₂ and diethyl pyrocarbonate were purchased from Sigma (London) Chemical Co., Poole, Dorset, U.K. Imidazole and 8-anilinoanthracene-1-sulphonate were purchased from BDH Chemicals, Poole, Dorset, U.K. Sephadex G-25 was purchased from Pharmacia (G.B.), London W5 5SS, U.K. ADP was purchased as the monopotassium salt from Boehringer, Lewes, Sussex, U.K.

Stock solutions (30–60 mM) of diethyl pyrocarbonate in ethanol were made up freshly each day and stored at 0°C. There was no significant decomposition of these stock solutions over a 6 h period, as judged by the rate of reaction of *N*-acetyl-L-histidine with a known excess of diethyl pyrocarbonate. Unless otherwise stated the reaction of creatine kinase with diethyl pyrocarbonate was performed at 20°C in 50 mM-sodium phosphate buffer at pH 6.1. The extent of ethoxycarbonylation of histidine groups was determined spectrophotometrically by using the

molar absorption coefficient for the *N*^ε-ethoxycarbonylhistidine derivative of 3.9×10^3 litre·mol⁻¹·cm⁻¹ at 242nm (Choong *et al.*, 1977). Control experiments with *N*-acetyl-L-histidine and diethyl pyrocarbonate showed that this value was appropriate under the conditions employed here. Although it is possible that the molar absorption coefficient may be somewhat different when the histidine group resides in a protein environment, it should be noted that Choong *et al.* (1977) also used radioactive labelling to determine the extent of modification and the results obtained by the two methods were very similar.

The treatment of modified enzyme with hydroxylamine was performed essentially as described by Miles (1977); the concentration of hydroxylamine used in these experiments was generally 0.35M.

Determinations of the numbers of reactive thiol groups in creatine kinase before and after modification by diethyl pyrocarbonate were performed by using Nbs₂ as described previously (Price & Hunter, 1976).

The rate of inactivation of creatine kinase by diethyl pyrocarbonate was studied by withdrawing samples from the reaction mixture at known times and diluting them into 50mM-glycine/NaOH buffer at pH8.4, containing 50mM-imidazole. Control experiments of the type described by Price (1979) showed that this procedure effectively stopped the reaction at the time of sampling. It was also shown that neither the imidazole in the dilution buffer nor the ethanol in the reaction mixtures [at final concentrations of 1% (v/v) or less] had any effect on the activity of unmodified enzyme during the course of the experiments.

The binding of ADP to unmodified and to modified enzyme was studied by the 8-anilidonaphthalene-1-sulphonate-fluorescence-quenching method as described by McLaughlin (1974).

Results

Reaction of creatine kinase with diethyl pyrocarbonate

Stoichiometry of histidine modification. The reaction between creatine kinase and diethyl pyrocarbonate led to the modification of a number of histidine groups in the enzyme. Thus with 300μM-reagent and 10μM-enzyme subunits, the end point of the reaction (reached after 1h) corresponded to the modification of 4.85 histidine groups per subunit, and there was no further reaction (as judged by the absorbance at 242nm) on addition of further portions of reagent at this time. The changes in the *A*₂₄₂ were completely reversed by incubation with 0.35M-hydroxylamine for 30min, indicating that no

disubstituted histidine derivatives were formed under these conditions (Miles, 1977). It appears therefore that up to five of the sixteen histidine groups per enzyme subunit (Watts, 1973) can be modified by diethyl pyrocarbonate.

Kinetics of histidine modification. The reactions between 10μM-enzyme (subunits) and various concentrations of diethyl pyrocarbonate were monitored by recording the increase in *A*₂₄₂ as a function of time. The second-order plot of these data was non-linear (Fig. 1a), indicating that the five histidine groups that can be modified were not all reacting at the same rate. A detailed analysis of the kinetics of the modification reaction to attempt to divide the groups into sets of different reactivity (Freedman & Radda, 1968) proved to be difficult. This difficulty almost certainly arose from complications caused by hydrolysis of diethyl pyrocarbonate during the reaction (Miles, 1977). The half-life of the reagent under the conditions of the modification reaction was found by determining the concentration of reagent remaining after given time periods by reaction with excess *N*-acetyl-L-histidine. The value obtained (25min) was comparable with those previously observed under similar conditions (Miles, 1977).

However, the non-linearity of the plot in Fig. 1(a) is unlikely to be caused by this hydrolysis reaction, but does indeed arise from non-identical reactivity of the histidine groups in the enzyme. Thus the second-order plot for reaction of the model compound *N*-acetyl-L-histidine (50μM) with diethyl pyrocarbonate (1mM) under these conditions was linear for at least 15min (Fig. 1b), and a second-order rate constant of 110M⁻¹·min⁻¹ was derived for this reaction.

Specificity of the reaction. Diethyl pyrocarbonate has been reported to react with a number of other groups in proteins and model compounds beside histidine, notably lysine, tyrosine and cysteine (Miles, 1977). In the case of creatine kinase there was no change in the *A*₂₇₈ on reaction of 10μM-enzyme subunits with 300μM-diethyl pyrocarbonate for 1h, indicating that no modification of tyrosine groups had occurred (Melchior & Fahrney, 1970; Miles, 1977).

There was, however, significant modification of the cysteine group on each subunit of the enzyme that is reactive towards Nbs₂. Thus after 10min of the reaction between 10μM-enzyme subunits and 300μM-diethyl pyrocarbonate the content of reactive cysteine groups had declined from 0.95 to 0.75 per subunit. After 40min reaction this value had declined further to 0.55 per subunit. The reaction between diethyl pyrocarbonate and cysteine groups has been previously noted in model systems (Larrouquère, 1965), although not in proteins (Miles, 1977).

There was also evidence for modification of a lysine group (or groups) in creatine kinase; this is

Fig. 1. Reactions of diethyl pyrocarbonate with creatine kinase and with *N*-acetyl-L-histidine

(a) Second-order plot for reaction of 10 μM -enzyme subunits with various concentrations of diethyl pyrocarbonate at 20°C in 50 mM-sodium phosphate buffer, pH 6.1. The concentrations of diethyl pyrocarbonate are 600 μM ($\circ$), 300 μM (Δ) and 150 μM ($\square$). On the ordinate, $f(x)$ is defined by:

$$f(x) = \frac{1}{A-B} \ln \left[\frac{B(A-x)}{A(B-x)} \right]$$

where A is the concentration of diethyl pyrocarbonate, B is the total concentration of histidine groups reacting (five per enzyme subunit) and x is the concentration of product formed at time t . (b) Second-order plot for reaction of 50 μM -*N*-acetyl-L-histidine with 1 mM-diethyl pyrocarbonate at 20°C in 50 mM-sodium phosphate buffer, pH 6.1. The significance of the ordinate is as in (a).

Table 1. Effects of ligands on the rate of inactivation of creatine kinase by diethyl pyrocarbonate

Enzyme subunits (10 μM) were allowed to react with 300 μM -diethyl pyrocarbonate at 20°C in 50 mM-sodium phosphate buffer, pH 6.1. The concentrations of ligands, when added, were: ADP, 5 mM; magnesium acetate, 6 mM; creatine, 40 mM; sodium nitrate, 100 mM.

Ligands present	Second-order rate constant ($\text{M}^{-1} \cdot \text{min}^{-1}$)	Protection (%)
None	740	0
Mg^{2+} + ADP	460	38
Mg^{2+} + ADP + creatine	370	50
Mg^{2+} + ADP + creatine + NO_3^-	110	85

could be analysed as a pseudo-first-order process for at least 80% of the total reaction (i.e. 20% of the original activity remaining). From this experiment the second-order rate constant was calculated to be 740 $\text{M}^{-1} \cdot \text{min}^{-1}$ (Table 1).

Effects of substrates on the kinetics of inactivation. The inclusion of combinations of substrates such as MgADP or MgADP plus creatine led to substantial degrees of protection against inactivation (Table 1). Protection was even more pronounced in the presence of MgADP plus creatine plus nitrate (Table 1), a combination that is thought to produce a complex resembling the transition state of the enzyme-catalysed reaction (Milner-White & Watts, 1971; Milner-White & Kelly, 1976). The results in Table 1 show that the 'transition-state-analogue' complex can afford about 85% protection against inactivation by diethyl pyrocarbonate.

Correlation of inactivation with modification of histidine groups. As noted previously modification of tyrosine groups in creatine kinase was not significant under the reaction conditions employed.

discussed below in the section dealing with the effects of hydroxylamine on the modified enzyme.

Kinetics of inactivation. The loss of enzyme activity on reaction with excess diethyl pyrocarbonate

Reaction of imidazole groups with diethyl pyrocarbonate can be reversed by treatment with hydroxylamine (Miles, 1977), and it has already been mentioned that for creatine kinase the increase in A_{242} was completely reversed by this treatment. The effect of hydroxylamine on the activity of the enzyme modified by diethyl pyrocarbonate was therefore studied to assess how much of the inactivation was due to reaction of histidine groups.

Enzyme that had been modified to the level of 25% residual activity was treated with 0.35M-hydroxylamine. The activity was restored to 70% of that of the control sample (to which hydroxylamine had been added) after 30min incubation. Use of higher concentrations of hydroxylamine (1M) or of longer periods of incubation (up to 1h) did not increase this level of re-activation. It must therefore be concluded that the loss of the 30% activity that is not restorable by hydroxylamine is due to modification of groups other than histidine. It has been noted (Melchior & Fahrney, 1970) that modification of lysine groups is not reversed by hydroxylamine, and Miles (1977) states that the same situation obtains for thiol compounds (e.g. cysteine). For creatine kinase, incubation of enzyme modified with diethyl pyrocarbonate (to the extent that the reactive cysteine content had declined from 0.95 to 0.68 per subunit) with 0.35M-hydroxylamine for periods of up to 30min did not regenerate any reactive cysteine groups. Since modification of this reactive cysteine group by a variety of reagents leads to inactivation of the enzyme (Watts, 1973; Bickerstaff & Price, 1978a), it is probable that part of the inactivation of creatine kinase by diethyl pyrocarbonate is due to modification of this cysteine group.

The conclusion from these experiments is that, although most of the observed inactivation of the enzyme by diethyl pyrocarbonate can be accounted for by modification of histidine groups, there are significant contributions from modification of lysine and cysteine groups. For the enzyme with 25% residual activity, the reactive cysteine content was 0.80 per subunit (compared with the initial value of 0.95) and thus it can be estimated that the loss of approx. 15% activity was due to modification of cysteine groups. Accordingly some modification experiments were also performed with enzyme in which the reactive cysteine groups had been reversibly protected by treatment with potassium tetrathionate, as described below.

Determination of the number of histidine groups essential for activity. In a situation in which a number of groups can be modified by a particular reagent, a detailed analysis is necessary to establish the exact number of these groups that are essential for activity. Two such methods of analysis have been described: these are based on a comparison of the rates of processes (Ray & Koshland, 1961) or on a comparison of the stoichiometry of processes (Tsou, 1962). The application of these methods to the present problem will be discussed in turn.

(a) Method of Ray & Koshland (1961). In this method the rate of inactivation is compared with the rates of modification of particular groups in order to establish which group or groups are essential for activity. As already mentioned, up to five histidine groups per creatine kinase subunit can be modified by diethyl pyrocarbonate, and although there is evidence (Fig. 1a) that these groups react at different rates, it was not possible to analyse the kinetic data to

Fig. 2. Reactions of modified creatine kinase with diethyl pyrocarbonate

Creatine kinase was modified in the absence and presence of the 'transition-state-analogue' complex to yield derivatives A and B respectively (the extents of modification of A and B were 1.55 and 0.80 histidine groups per subunit respectively). (a) Derivatives A and B (at concentrations of $10 \mu\text{M}$ -subunits) were then allowed to react with $300 \mu\text{M}$ -diethyl pyrocarbonate at 20°C in 50mM -sodium phosphate buffer, pH 6.1: $\circ$, derivative A; Δ , derivative B; $\square$, difference between reactions of B and A. (b) Semilogarithmic plot of the difference between reactions B and A in (a). On the ordinate, P_{∞} represents the limiting difference (0.72 histidine group per subunit) and P_t the difference observed at time t .

determine the rate of modification of any particular group or set of groups. An alternative solution to this problem was therefore devised as described below.

The presence of the 'transition-state-analogue' complex leads to a high degree of protection against inactivation (Table 1). The extent to which this complex protected against modification of histidine groups could not be estimated directly, because the ADP present masked any changes in the A_{242} on reaction. However, it was possible to estimate the extent of histidine modification by measuring the decrease in the A_{242} of a sample of modified enzyme

on addition of 0.35M-hydroxylamine, provided that the sample had been gel-filtered to remove ligands (such as ADP, creatine and nitrate) and excess reagent. A control experiment showed that this method gave results in good agreement with the direct method (based on the increase in the A_{242}) in the case of modification of enzyme in the absence of ligands. The changes in A_{242} on addition of hydroxylamine were monitored over a 30 min period, after which time no further changes occurred.

With this method it was possible to show that reaction of $49\mu\text{M}$ -enzyme subunits with $300\mu\text{M}$ -diethyl pyrocarbonate for 8 min led to modification of 1.55 histidine groups per subunit and a loss of 81% of the initial activity. In the presence of the 'transition-state-analogue' complex the extent of modification under these conditions was decreased to 0.80 group per subunit and the loss of activity to 24%. It is thus clear that the complex protects 0.75 histidine group per subunit from reaction. These two samples were then taken and subjected to further reaction with diethyl pyrocarbonate with the results shown in Fig. 2(a). The difference between these subsequent reactions (Fig. 2a) reaches a limit of 0.72 histidine group per subunit and the rate constant, calculated from the semilogarithmic plot (Fig. 2b), is $580\text{M}^{-1}\cdot\text{min}^{-1}$.

The conclusions from these experiments are that the presence of the 'transition-state-analogue' complex prevents nearly one histidine group per subunit from reacting with diethyl pyrocarbonate and that the rate constant for modification of this group (determined by the difference between the subsequent modification reactions) is $580\text{M}^{-1}\cdot\text{min}^{-1}$. This value is similar to the rate constant for the inactivation of the enzyme by diethyl pyrocarbonate ($740\text{M}^{-1}\cdot\text{min}^{-1}$).

Fig. 3. Enzyme activity remaining as a function of extent of histidine modification

Enzyme samples were allowed to react with various concentrations of diethyl pyrocarbonate (up to $300\mu\text{M}$) for various periods of time (up to 30 min) before samples were taken for assay. The extents of modification of histidine groups were determined spectrophotometrically as described in the text.

Fig. 4. Plots of the data in Fig. 3 by the method of Tsou (1962)

The significance of the axes is described in the text. (a) Plots made assuming $i = p = 1$ ($\circ$) or $i = 1, p = 2$ (Δ). (b) Plot made assuming $i = p = 2$.

It would be expected that the rate constant for modification of this histidine group would be somewhat lower than that for inactivation, since it has already been shown that modifications of lysine and cysteine groups also contribute to the loss of activity.

(b) Method of Tsou (1962). In this method, an analysis is made of data correlating the extent of inactivation with the extent of modification of a particular group or groups. The data are plotted graphically making different assumptions about the number of essential groups and their division into sets of different reactivity. From these plots the most satisfactory set of assumptions can be derived. The method has been discussed in more detail by other workers, e.g. Paterson & Knowles (1972) and Norris & Brocklehurst (1976). Fig. 3 shows the data obtained for the modification of creatine kinase by diethyl pyrocarbonate in a number of experiments in which different concentrations of reagents or different periods of time of reaction were employed. Although extrapolation from the data points at relatively low extents of modification can be made to suggest that modification of one histidine group per subunit leads to complete inactivation, it is clear that data points at higher extents of modification deviate markedly from this line.

The case considered by Tsou (1962) that is most appropriate to this situation is that in which the essential and non-essential groups react at significantly, but not markedly, different rates. The equation derived (Tsou, 1962) is:

$$\log \left(\frac{nx}{a^{1/i}} - p \right) = \log(n-p) + \left(\frac{\alpha-1}{i} \right) \log a$$

where n = total number of groups modified (five in this case), a = activity (fractional) remaining after modification of $n(1-x)$ groups, α = ratio of rate constants (slow set/fast set) and p = number of groups in a fast

set of which i are essential. A plot of $\log \left(\frac{nx}{a^{1/i}} - p \right)$

against $\log a$ should give a straight line of slope $\left(\frac{\alpha-1}{i} \right)$ and intercept $\log(n-p)$. The data in Fig. 3

were plotted according to this equation making various sets of assumptions, e.g. $i=p=1$; $i=1$, $p=2$; $i=p=2$ etc. Fig. 4 shows the plots for these cases, and it can be seen that a satisfactory linear plot is obtained when $i=p=1$, but not in the other cases. (Other sets of assumptions, e.g. $i=p=3$, also give non-linear plots.) From the slope of the plot when $i=p=1$, it can be deduced that $\alpha=0.16$.

The conclusion from this type of analysis is that there is one essential histidine, in agreement with the conclusions from the analysis by Ray & Koshland (1961).

Reaction of thiol-protected creatine kinase with diethyl pyrocarbonate

Since there was clear evidence that diethyl pyrocarbonate could modify the reactive cysteine groups of creatine kinase, it was decided to perform some experiments with thiol-protected enzyme in which case the specificity of the modification reaction should be improved. The enzyme was inactivated by treatment with a 100-fold excess of potassium tetrathionate (expressed relative to the subunit concentration) for 10 min at 0°C; complete restoration of activity was achieved by incubation with 7 mM-dithiothreitol for 10 min at 0°C. These findings are essentially identical with those reported by Kassab *et al.* (1968).

Characterization of the modification reaction. Experiments analogous to those described for unprotected creatine kinase showed that up to five histidine groups per subunit in the protected enzyme could be modified by diethyl pyrocarbonate. Since the changes in the A_{242} could be completely reversed by treatment with 0.35 M-hydroxylamine for 30 min, it was concluded that no disubstituted histidine derivatives had been formed (Miles, 1977). There was no evidence for changes in the A_{278} during the reaction, indicating that modification of tyrosine groups was not significant. Modification of the reactive cysteine groups in the enzyme was prevented by the reversible blocking procedure.

Experiments designed to test the effect of hydroxylamine on the activity of modified protected enzyme gave evidence for a small amount of modification of lysine groups by diethyl pyrocarbonate. Protected enzyme was allowed to react with diethyl pyrocarbonate to the extent of 30% residual activity. (It should be noted that this activity is measured after incubation of a diluted sample for 10 min in 50 mM-glycine/NaOH buffer, pH 8.4, containing 50 mM-imidazole and 7 mM-dithiothreitol, in order to remove the thiol-protecting group from the enzyme.) Treatment of this modified enzyme with 0.35 M-

Table 2. *Effects of ligands on the rate of inactivation of thiol-protected creatine kinase by diethyl pyrocarbonate*
Enzyme subunits (6.8 μ M) were allowed to react with 300 μ M-diethyl pyrocarbonate. The concentrations of ligands and other conditions are given in the legend to Table 1.

Ligands present	Second-order rate constant ($M^{-1} \cdot min^{-1}$)	Protection (%)
None	470	0
Mg ²⁺ + ADP	380	19
Mg ²⁺ + ADP + creatine	350	26
Mg ²⁺ + ADP + creatine + NO ₃ ⁻	350	26

hydroxylamine for 30min restored the activity to 90% of the control value; this level of re-activation could not be increased by use of higher concentrations of hydroxylamine (up to 1M) or longer periods of incubation (up to 1 h). It was concluded that the 10% of the activity that could not be restored by hydroxylamine treatment was due to modification of lysine groups by diethyl pyrocarbonate.

Kinetics of inactivation. The protected enzyme was allowed to react with diethyl pyrocarbonate under conditions analogous to those used for the unprotected enzyme (Table 1). The rate constants for reactions conducted in the absence and presence of various ligands are collected in Table 2. The combinations MgADP and MgADP plus creatine afford some protection against inactivation, but the

combination MgADP plus creatine plus nitrate affords no further protection. This lack of extra protection can be accounted for by the fact that the protected enzyme probably cannot form the 'transition-state-analogue' complex; further studies have emphasized the need for the integrity of the reactive thiol groups in the formation of this complex (Keighren & Price, 1978).

Correlation of the extent of inactivation with the extent of histidine group modification. Data from a number of experiments in which protected enzyme was allowed to react with different concentrations of diethyl pyrocarbonate for different periods of time are summarized in Fig. 5. As in the case of unprotected enzyme (Fig. 3), it is not possible from this plot to deduce the number of essential histidine groups. An analysis of the data in Fig. 5 was undertaken by the method of Tsou (1962) by using the equation already given. The appropriate plots for the assumptions $i = p = 1$, $i = 1, p = 2$ and $i = p = 2$ are shown in

Fig. 5. Enzyme activity remaining as a function of extent of histidine modification for thiol-protected enzyme. Experiments were performed as described in the legend to Fig. 3.

Table 3. Dissociation constants for enzyme-ADP complexes. Dissociation constants were determined by the probe-fluorescence-quenching method (McLaughlin, 1974). Experiments were performed at 25°C in 50mM-sodium phosphate buffer, pH 6.1. Modified enzyme had been allowed to react with diethyl pyrocarbonate to the extent of 5% residual activity (2.45 histidine groups per subunit modified).

Ligands present	K_d (μ M)	
	Unmodified enzyme	Modified enzyme
Magnesium acetate (5 mM)	130	80
Magnesium acetate (5 mM) + creatine (40 mM)	110	90

Fig. 6. Plots of the data in Fig. 5 by the method of Tsou (1962). The significance of the axes is described in the text. (a) Plots made assuming $i = p = 1$ (○) or $i = 1, p = 2$ (Δ). (b) Plot made assuming $i = p = 2$.

Fig. 6; the data are fitted most satisfactorily by assuming that there is one essential histidine group constituting a fast-reacting set. (Other plots made by using different assumptions, e.g. $i = p = 3$, were grossly non-linear and are not shown in Fig. 6.)

The conclusion from this analysis is that there is one essential histidine group in the protected enzyme, in agreement with the conclusions reached for the unprotected enzyme.

Binding of ADP to the modified enzyme

The binding of nucleotide to the enzyme was studied by the anilidonaphthalenesulphonate-fluorescence-quenching method (McLaughlin, 1974). Values for the dissociation constants obtained at 25°C in 50 mM-sodium phosphate buffer, pH 6.1, are summarized in Table 3 for unmodified enzyme and for enzyme allowed to react with diethyl pyrocarbonate to the extent of 2.45 histidine groups modified per subunit (5% residual activity). The modified enzyme is able to bind ADP both in the presence of Mg^{2+} and of Mg^{2+} plus creatine with an affinity similar to or slightly greater than that of unmodified enzyme. This conclusion is consistent with that reached by Roustan *et al.* (1970), who reported that modified creatine kinase exhibited a similar difference spectrum to that shown by unmodified enzyme on addition of substrates (MgATP or MgADP).

Discussion

The experiments described in this paper have explored a number of aspects of the reaction between creatine kinase and diethyl pyrocarbonate. The principal conclusion that there is one essential histidine group per enzyme subunit, modification of which leads to loss of activity, is in agreement with the conclusion by Pradel & Kassab (1968). However, a number of new aspects of the reaction have been investigated.

Firstly, Pradel & Kassab (1968) did not investigate in detail the characteristics of the modification reaction. Our results show that a number of histidine groups (up to five per subunit) can be modified, and that there is no evidence for formation of disubstituted histidine derivatives or for modification of tyrosine groups. There is a small but significant contribution to the inactivation from modification of the reactive cysteine groups and of lysine groups. The experiments with the thiol-protected enzyme provide a more clear-cut picture, since the reactive cysteine groups are prevented from reacting with diethyl pyrocarbonate, and the level of re-activation by hydroxylamine is correspondingly greater in this case.

Secondly, our studies have shown in more detail the extent of protection afforded by substrates against inactivation. In particular, the combination MgADP plus creatine plus nitrate, which is thought

to produce a 'transition-state-analogue' complex, affords a very high degree of protection against inactivation (Table 1). The enhanced protection in this case might arise from the tighter binding of substrates to the enzyme in this complex (Watts, 1973), or from the conformational change in the enzyme (Keighren & Price, 1978), which could lead to decreased reactivity of the histidine group. At present these two possibilities cannot be distinguished. Whatever the cause, however, it is shown that the complex affords protection against modification of nearly one histidine group per subunit. The rate constant for modification of this histidine group can be deduced by subsequent reaction with excess reagent (Fig. 2).

Thirdly, our studies have made more detailed analyses of the data to indicate the number of essential histidine groups in the enzyme. The results of the kinetic method of Ray & Koshland (1961) and the stoichiometric method of Tsou (1962) both show that the most satisfactory explanation of the data is that there is only one essential histidine group per subunit, even though a number of other histidine groups react at a lower rate.

In summary, these experiments emphasize the need for a careful scrutiny of the specificity of a chemical-modification reaction and for a detailed analysis in order to draw conclusions about the number of essential groups involved, especially in cases where a number of groups can be modified. Although the results of the experiments here (particularly on substrate protection and on the stoichiometry of modification) can be explained in terms of a histidine group at the active site of the enzyme, this conclusion cannot be considered unequivocal in the absence of results from other studies such as X-ray crystallography or affinity labelling. Nevertheless the results are consistent with a histidine group playing an important role in the mechanism of the enzyme, and it is possible that this role could be that of a proton-donating/accepting species (Allen & Haake, 1976; Bickerstaff & Price, 1978a).

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