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Selective Modification of Rabbit Muscle Pyruvate Kinase by 5-Chloro-4-oxopentanoic Acid

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Rabbit muscle pyruvate kinase was irreversibly inactivated by 5-chloro-4-oxopentanoic acid with a pK_a of 9.2. The inhibition was time-dependent and was related to the 5-chloro-4-oxopentanoic acid concentration. Analysis of the kinetics of inhibition showed that the binding of the inhibitor showed positive co-operativity ($n = 1.5 \pm 0.2$). Inhibition of pyruvate kinase by 5-chloro-4-oxopentanoic acid was prevented by ligands which bind to the active site. Their effectiveness was placed in the order Mg^{2+} > phosphoenolpyruvate >ATP$\gg$ADP>pyruvate. Inhibitor-modified pyruvate kinase was unable to catalyse the detritiation of [3-^{3}H]pyruvate in the ATP-promoted reaction, but it did retain 5–10% of the activity with either phosphate or arsenate as promoters. 5-Chloro-4-oxo-[3,5-^{3}H]pentanoic acid was covalently bound to pyruvate kinase and demonstrated a stoicheiometry of 1 mol of inhibitor bound per mol of pyruvate kinase protomer. The incorporation of the inhibitor and the loss of enzyme was proportional. These results are discussed in terms of 5-chloro-4-oxopentanoic acid alkylating a functional group in the phosphoryl overlap region of the active site, and a model is presented in which this compound alkylates an active-site thiol in a reaction that is controlled by a more basic group at the active site.

Many advances in protein chemistry can be attributed to the use of reagents that specifically modify proteins (Cohen, 1970; Shaw, 1970). The most common of the reagents used for protein alkylation have been of general structure X–CH_2–COR, where X is a halogen. Either iodoacetate or iodoacetamide are usually used for alkylation experiments. Because of the success of this approach we decided to synthesize a variety of alkylating probes based on the structure Cl–CH_2–CO–$[CH_2]_n$–COR.

As an initial report to this project, we have analysed the reaction of 5-chloro-4-oxopentanoic acid and its analogues with pyruvate kinase (ATP–pyruvate phosphotransferase, EC 2.7.1.40). Pyruvate kinase is inactivated by reagents that alkylate both cysteine thiol (Jacobson & Black, 1971; Flashner *et al.*, 1972) and lysine ε-NH_2 groups (Hollenberg *et al.*, 1971), and it was hoped that the interaction of 5-chloro-4-oxopentanoic acid with the protein might give further information on the role of these groups in the catalytic reaction. Further, we were aware that bromopyruvate reacts non-specifically with pyruvate kinase (Flashner *et al.*, 1972), which gave us the opportunity to evaluate whether the chloromethyl ketone group confers a greater degree of specificity in the alkylation reaction.

In this communication, we show that 5-chloro-4-oxopentanoic acid specifically alkylates a single site on pyruvate kinase and that this site may be located in the vicinity of the phosphoryl overlap region between ATP and phosphoenolpyruvate (Reynard *et al.*, 1961).

Materials and Methods

Synthesis of 5-chloro-4-oxopentanoate

β-Carboxymethylpropionyl chloride was prepared by the method of Cason (1955). Diazomethane was prepared from 35.2 g of *p*-tolylsulphonylmethylnitrosamide and collected in 400 ml of diethyl ether, which was then dried over KOH pellets for 16 h at 4°C. The solution was transferred to a three-necked flask equipped with dropping funnel, stirrer, gas inlet and ice bath. Then 10 ml of freshly distilled β-carboxymethylpropionyl chloride was added dropwise over 20 min. The mixture was allowed to stir for 1 h. The reaction was shown to be complete by i.r. spectroscopy which showed the appearance of the diazonium ion at 2210 cm^{-1}, a new carbonyl absorption at 1650 cm^{-1} and complete loss of the acid chloride carbonyl at 1810 cm^{-1}. Ester absorption was maintained at 1740 cm^{-1}.

The mix was now connected to an HCl gas source

(conc. H_2SO_4 dropped into conc. HCl is superior to an HCl cylinder) and gas was passed for 30min with continued stirring at 4°C. The colourless solution was then washed with 6×100ml of satd. NaCl, and the aqueous phase was back-extracted with 5×100ml of diethyl ether. The pooled ethereal fraction was dried over anhydrous Na_2SO_4 and evaporated to dryness *in vacuo*. The residual material was purified by rapid vacuum distillation to yield 7.8g of 5-chloro-4-oxopentanoate methyl ester [b.p. 88–92°C at 80Pa (0.6mmHg); n.m.r. gives δ 4.2p.p.m., 2H, singlet, Cl–$\underline{H}_2$–; δ 3.65p.p.m., 3H, singlet, –$CO_2C\underline{H}_3$; δ 2.6 to 2.9p.p.m., 4H, multiplet, –$C\underline{H}_2$–$C\underline{H}_2$–].

The methyl ester (4.4g) was hydrolysed in 12ml of 7M-HCl for 30h at 20°C and evaporated to dryness *in vacuo*. The residue was dissolved three times in water and dried again to remove traces of HCl. It was finally evaporated to dryness three times from benzene to remove water. The product was crystallized from diethyl ether/light petroleum (b.p. 60–80°C), yielding 2g of 5-chloro-4-oxopentanoic acid (m.p. 68–70°C; n.m.r. gives δ 4.2p.p.m., 2H, singlet, Cl–$C\underline{H}_2$–; δ 2.7 to 3.15p.p.m., 4H, multiplet, –$C\underline{H}_2$–$C\underline{H}_2$–).

Synthesis of 5-chloro-4-oxo[3,5-^{3}*H*]*pentanoic acid*

5-Chloro-4-oxopentanoic acid (160mg) was dissolved in 1ml of ^3H_2O (1Ci) containing 20mM-HCl, placed in a sealed evacuated tube and heated at 110°C and 103kPa (15lb/in^2) for 1h. The product was freeze-dried and the ^3H_2O was collected. Excess of radioactivity was removed by dissolving in water and freeze-drying. This was repeated three times. The product was dried by evacuation of benzene/methanol (4 times) and diethyl ether (twice) before crystallization (yield, 80mg). To confirm radiochemical purity the compound was converted into its methyl ester with diazomethane in diethyl ether. G.l.c. on a polydiethyleneglycol succinate column at 190°C by using a Panax radiogas detector system showed the compound had the same retention time as the authentic compound (7.8min) and revealed only a single radioactive peak.

Since ^3H is inserted into a labile position, specific-radioactivity determinations were designed to eliminate any ^3H_2O. Some 4mg of the labelled alkylating agent was dissolved in methanol, and a sample removed to determine its total ^3H content. Then radioactivity was stabilized with $NaBH_4$ (1mg; 1h reaction). Methanol was removed *in vacuo* and the evaporation to dryness from methanol was repeated four times. The product was dissolved in methanol and its ^3H content determined again. Usually, at least 95% of ^3H inserted into 5-chloro-4-oxopentanoic acid was found to be stable.

Pyruvate kinase

Ten preparations of rabbit muscle pyruvate kinase were used in the present work. Five of the preparations were obtained from a commercial source, whereas the remainder were purified by the method of Tietz & Ochoa (1962). One of these was further purified by chromatography on Whatman CM-52 CM-cellulose (Stammers & Muirhead, 1975). All of these preparations showed the same reaction properties with 5-chloro-4-oxopentanoic acid, but we do find that $k_{app.}$ for inactivation varies by a factor of two. No apparent reason for this has been observed. When native pyruvate kinase was used, protein concentration was determined by using an extinction coefficient at 280nm of 30×10^3 litre·mol^{-1}·cm^{-1} (Bucher & Pfleiderer, 1955). However, with modified protein kinase, protein was measured with Folin–Ciocalteu reagent (Lowry *et al.*, 1951).

For small-scale alkylation reactions, 50μg of pyruvate kinase was dissolved at 20°C in 1ml of 0.1M-Tris/HCl, pH9.2, containing 100mM-KCl, 1mM-EDTA and the appropriate concentration of 5-chloro-4-oxopentanoic acid. At suitable reaction times a small sample was removed and pyruvate kinase activity was determined by the procedure of Bucher & Pfleiderer (1955). First-order rate constants for inactivation by this compound were determined from the slope of plots of log (percentage activity) against inactivation time. 5-Chloro-4-oxopentanoic acid was usually dissolved in water and neutralized with $KHCO_3$. The solution cannot be stored and is unstable at extremes of alkali.

When pyruvate was included in the alkylation mixture, the lactate dehydrogenase-coupled assay for pyruvate kinase could not be used. For this assay, the reaction was followed by measuring the loss of phosphoenolpyruvate at 230nm in a reaction mixture consisting of 1ml of 25mM-Tris/HCl, pH7.4, 100mM-KCl, 400μm-ADP, 400μM-phosphoenolpyruvate, 2.5mM-$MgCl_2$ and enzyme.

[3-^{3}*H*]*Pyruvate and pyruvate kinase-promoted detritiation*

Sodium pyruvate (30mg) was dissolved in 0.5ml of ^3H_2O (0.5Ci) and autoclaved at 103kPa (15lb/in^2) for 30min. [3-^{3}H]Pyruvate was purified on a column (15cm×0.7cm) of Dowex 1 (Cl^- form) by using a 100ml gradient from 0 to 0.1M-HCl. Pyruvate was located by assay with lactate dehydrogenase, adjusted to pH6.2 with NaOH and freeze-dried (yield, 16mg; 9.5×10^6 d.p.m./μmol).

For detritiation assays 0.05μkat of pyruvate kinase (approx. 10μg of protein) were incubated in 0.20ml of 50mM-Tris/HCl, pH8.0, containing 50mM-KCl, 5mM-$MgCl_2$, 4.3mM-[3-^{3}H]pyruvate (1.25×10^6 d.p.m./μmol) and the appropriate 'phos-

phoryl' promoter of detritiation (ATP, P_i, arsenate). After 30min, ^3H lost as ^3H_2O was determined as the radioactivity that was not retained by a column (3cm×0.7cm) of Dowex 1 (Cl^- form).

Source of materials

Rabbit muscle pyruvate kinase, pig heart lactate dehydrogenase, phosphoenolpyruvate, ATP and NADH were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. ^3H_2O was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Sephadex G-25 and G-10 were bought from Pharmacia Fine Chemicals, Uppsala, Sweden. All other chemicals were from either BDH, Poole, Dorset, U.K., or Koch-Light Laboratories, Colnbrook, Bucks., U.K.

Results

Characteristics of the alkylation reaction

To assess the alkylating properties of 5-chloro-4-oxopentanoic acid, rabbit muscle pyruvate kinase was incubated in a buffer with an ionic composition similar to the usual assay solution, 50mM-Tris/HCl containing 100mM-KCl and 1mM-EDTA, at a variety of pH values in the presence of a fixed concentration of the alkylating agent (10mM). At appropriate times over a 1h incubation, samples were removed for assay and showed that the 5-chloro-4-oxopentanoic acid-treated enzyme lost activity progressively in comparison with the control enzyme. A plot of log (percentage activity) versus incubation time demonstrated that inactivation followed pseudo-first-order kinetics and enabled the determination of an apparent first-order rate constant ($k_{app.}$). Fig. 1 demonstrates that $k_{app.}$ is pH-dependent. A plot of $k_{app.}^{-1}$ versus [H^+] enabled the estimation of the pK_a for the alkylation reaction as 9.2 and $k_{app.}^*$ as 0.23min^{-1}. The solid line in Fig. 1 is the variation of $k_{app.}$ obtained by using the predicted values of pK_a and $k_{app.}^*$ and shows good agreement with the experimental points. A simple pK_a for the alkylation reaction suggests that 5-chloro-4-oxopentanoic acid might be titrating a single group on the enzyme. Further, the relative high pK_a value might indicate the participation of an unprotonated amino group of lysine in the alkylation reaction. This statement is amplified by two observations. First, the pK_a for alkylation of pyruvate kinase thiols by 5,5′-dithiobis-(2-nitrobenzoate) (pK_a, 7.4; Flashner *et al.*, 1972) or iodoacetamide (pK_a 6.8 and 7.8; Jacobson & Black, 1971) is substantially lower than 9.2. Secondly, titration of the cysteine thiol with 5-chloro-4-oxopentanoic acid, as monitored by the disappearance of 5,5′-dithiobis-(2-nitrobenzoate)-reacting material, revealed that the pK_a for this reaction was also substantially lower than that for the enzyme inactivation.

For active-site-directed inhibitors it is generally assumed that the inhibitor forms a Michaelis-type complex (EI) with the enzyme before covalent reaction (EI*) as follows:

$$E+I \underset{}{\overset{K_I}{\rightleftharpoons}} EI \xrightarrow{k_r} EI^*$$

The loss of activity in this system is given by eqns. (1) (Kitz & Wilson, 1962):

$$\ln\frac{E-EI^*}{E}=\frac{-k_r t}{1+K_{1/I}}=-k_{app.}\cdot t \qquad (1)$$

It follows that inactivation should follow pseudo-first-order kinetics and that $k_{app.}$ should be a function of inhibitor concentration. Fig. 2 demonstrates that this is the case for the inactivation of pyruvate kinase by 5-chloro-4-oxopentanoic acid. Normally values of K_I and k_r can be obtained from these data by a double-reciprocal plot of $k_{app.}$ versus inhibitor concentration. However, when the data from Fig. 2 were analysed by this procedure (by using an unweighted least-squares hyperbolic regression

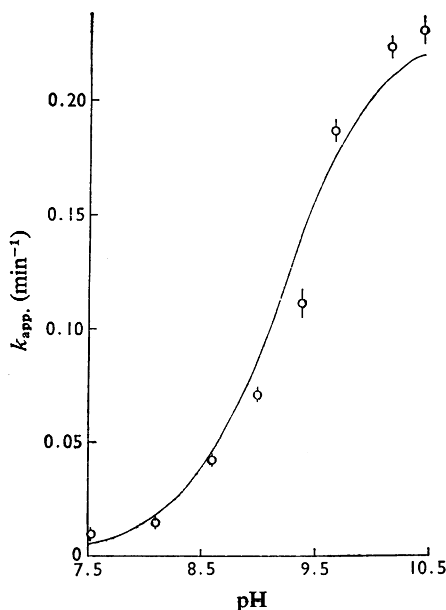


Fig. 1. *pH-dependence of the rate constant for inactivation of pyruvate kinase by 5-chloro-4-oxopentanoate*

The alkylation reaction was carried out in 10mM-5-chloro-4-oxopentanoate, 50mM-Tris/HCl, 100mM-KCl and 1mM-EDTA at a number of pH values and the first-order rate constant for inactivation ($K_{app.}$) was estimated. The solid line shows the calculated result for pK_a 9.2 and $k_{app.}$ at zero H^+ ($k_{app.}^*$) as 0.23min^{-1}. No pH-dependent inactivation of pyruvate kinase was observed in the absence of the inhibitor. The bars indicate the standard error for each experimental point.

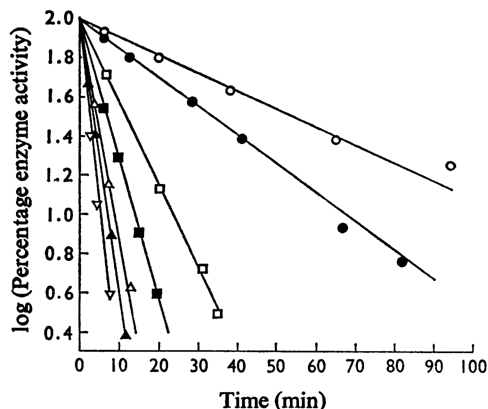


Fig. 2. *5-Chloro-4-oxopentanoate concentration-dependence in the inactivation of pyruvate kinase*

The inactivation of pyruvate kinase was measured under the standard reaction conditions described in the Materials and Methods section, in the presence of various concentrations of 5-chloro-4-oxopentanoate: ○, 1mM; ●, 2mM; □, 4mM; ■, 6mM; △, 8mM; ▲, 12mM; ▽, 20mM.

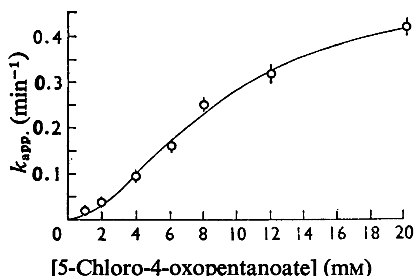


Fig. 3. *Co-operative binding of 5-chloro-4-oxopentanoate in the inactivation of pyruvate kinase*

The data from Fig. 2 are replotted (○). These data were used to calculate K_1 (9.3mM), k_r (0.54min^{-1}) and n (1.72) by using the Atkins (1972) analytical procedure for estimating parameters of the Hill equation. The solid line shows the predicted result for these values.

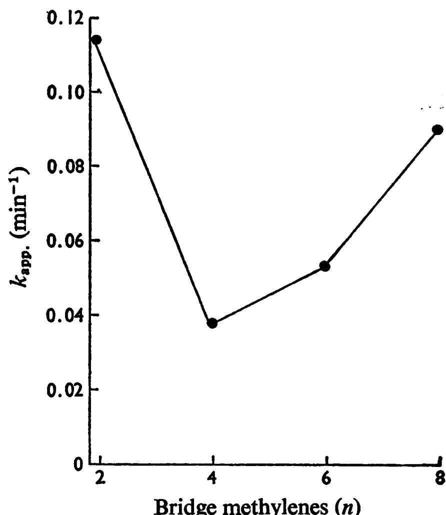
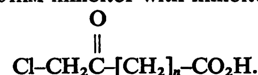


Fig. 4. *Effect of inhibitor chain length on the rate of inactivation of pyruvate kinase*

The rate constant for inactivation of pyruvate kinase was determined at 10mM inhibitor with inhibitors of general structure:



The plot shows the effect of varying the number of bridge methylenes (n).

analysis, Wilkinson, 1961) then only a poor fit was obtained. This divergence was due to the fact that binding of the inhibitor appeared to be sigmoidal. Analysis of the data in Fig. 2 by a Hill plot gave $k_r = 0.54\,min^{-1}$, $K_1 = 9.30\,mM$ and $n = 1.72$ (Fig. 3). The value of n has varied with the enzyme preparation and the average value is 1.50 ± 0.2. The apparent sigmoidal curve for inactivation by 5-chloro-4-oxopentanoic acid might be due either to co-operativity in binding or to significant decay of the inhibitor at low concentrations in the presence of traces of NH_4^+ ions at alkaline pH. The value for K_1 observed with 5-chloro-4-oxopentanoic acid compares reasonably with estimates for the K_m of pyruvate at 4.87mM when the enzyme is assayed in the direction of phosphoenolpyruvate formation (Giles *et al.*, 1975). Pyruvate kinase shows some flexibility in the chain-length specificity of the substrate (Bondinell & Sprinson, 1970; Soling *et al.*, 1971; Woods *et al.*, 1972). To test the preference in the alkylation reaction, we compared the potency of four related chloromethylketone fatty acids, namely 5-chloro-4-oxopentanoate, 7-chloro-6-oxohexanoate, 9-chloro-8-oxononanoate and 11-chloro-10-oxoundecanoate. There is no clear preference in inhibitory potency (Fig. 4). Thus $k_{app.}$ for a fixed concentration of inhibitor decreases to a minimum around seven carbon chain length. An observation of this type suggests that the ability to enter and modify the active site might in fact be a function of two characteristics, namely molecular size and hydrophobic character. Potentially these two facets might compensate one another to produce the bell-shaped curve in Fig. 4.

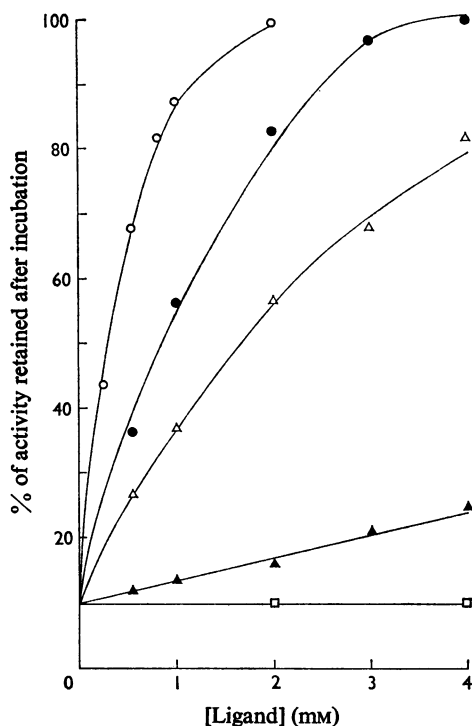


Fig. 5. *Protection of pyruvate kinase against inactivation by 5-chloro-4-oxopentanoate by using ligands that participate in the catalytic reaction*

Pyruvate kinase was allowed to react for 50min with 5mM-5-chloro-4-oxopentanoate under standard reaction conditions in the presence of various concentrations of added ligand and the loss of activity was estimated. The protecting ligands were: $MgCl_2$ (○); phosphoenolpyruvate (●); ATP (△); ADP (▲); pyruvate (□).

Protection characteristics

To investigate the potential site of action of 5-chloro-4-oxopentanoate, the ability of ligands that participate in the catalytic reaction to protect against inactivation by this compound was tested. The buffer chosen for these experiments was 50mM-Tris/HCl, 100mM-KCl and 1mM-EDTA, pH9.2. K^+ is obligatory in the normal catalytic reaction (Boyer *et al.*, 1942) and is known to protect pyruvate kinase from inactivation by iodoacetamide (Jacobson & Black, 1971). However, its presence or absence had no influence on the rate of alkylation by 5-chloro-4-oxopentanoate and it was decided to retain the cation in all subsequent work in order that the alkylation reaction medium retained the ionic composition of the catalytic medium despite the pH difference.

Five ligands were tested for their ability to protect against inactivation and it was established that ligands which form complexes at the active site do protect against alkylation, indicating that 5-chloro-4-oxopentanoate may be an active-site-directed reagent. The preference for protection could be placed in the order, Mg^{2+} > phosphoenolpyruvate > ATP ≫ ADP > pyruvate (Fig. 5). Despite the high pK_a for the alkylation reaction, which led us to predict the potential involvement of a lysine ε-NH_2 residue, the protection pattern is more consistent with experiments using thiol-directed reagents [i.e. 5,5′-dithiobis-(2-nitrobenzoate); Flashner *et al.* (1972)] as opposed to ε-NH_2-directed compounds (i.e. 2,4,6,-trinitrobenzene-1-sulphonate; Hollenberg *et al.*, 1971). The critical difference is that ADP offers excellent protection against 2,4,6,-trinitrobenzene-1-sulphonate (Hollenberg *et al.*, 1971), whereas it only affords marginal protection against 5-chloro-4-oxopentanoate or 5,5′-dithiobis-(2-nitrobenzoate). If 5-chloro-4-oxopentanoate does not bind within the nucleotide-binding region of the active site then surely it will bind in the alternative half as occupied by either phosphoenolpyruvate or pyruvate. Certainly phosphoenolpyruvate affords excellent protection against inactivation; however, quite unexpectedly, pyruvate does not prevent inactivation. Therefore it is clear that, although 5-chloro-4-oxopentanoate is an active-site-directed inhibitor, its mode of binding must be substantially different from pyruvate, of which it is supposedly an analogue. If the pattern of protection is analysed in terms of a rigid active site then it seems most logical that 5-chloro-4-oxopentanoate may alkylate a functional group in the vicinity of the phosphoryl overlap region of the active site (i.e. γ-phosphate in ATP or phosphate in phosphoenolpyruvate) (Reynard *et al.*, 1961). An alternative to this analysis is that 5-chloro-4-oxopentanoate may alkylate a group in the vicinity of the active site and the reactivity of this group may be influenced by conformational changes in the protein induced by ligands binding to the active site. Certainly, pyruvate kinase undergoes substantial conformational changes on binding ligands such as bivalent metal ions (Suelter & Melander, 1963).

Detritiation of [3-^{3}H]*pyruvate by modified and control enzyme*

Having failed to demonstrate protection by pyruvate against 5-chloro-4-oxopentanoate inactivation, we wanted to show that once the enzyme was alkylated then the pyruvate site was no longer available to the substrate. The simplest reaction to study from this point is to look at the detritiation of [3-^{3}H]pyruvate, a reaction that requires all the components of the catalytic reaction but does not necessarily involve overall catalysis (Rose, 1960).

Table 1. *Detritiation of* [3-^{3}*H*]*pyruvate by 5-chloro-4-oxopentanoate-modified pyruvate kinase*

Modified or control pyruvate kinase (30μg of protein) was incubated for 30min at 37°C in 0.20ml of 50mM-Tris/HCl, pH8, containing 5mM-[3-^{3}H]pyruvate (1.25×10^{3} d.p.m./nmol), 5mM-$MgCl_2$, 50mM-KCl and an appropriate detritiation promoter.

	$10^{-4}\times{}^{3}H_2O$ (d.p.m.)	
Detritiation promoter	5-Chloro-4-oxo-pentanoate-labelled pyruvate kinase	Control pyruvate kinase
None	1.5	1.5
ATP (2.5mM)	1.7	35.4
Phosphate (50mM)	3.1	21.0
Arsenate (50mM)	2.4	23.6

To modify sufficient enzyme for these experiments, 1mg of pyruvate kinase was incubated at 20°C in 3ml of 50mM-Tris/HCl buffer, pH9.4, containing 100mM-KCl and 1mM-EDTA either in the presence or absence of 10mM-5-chloro-4-oxopentanoate. After 2.5h, the control enzyme was unaffected, whereas the inhibitor-treated enzyme had lost all its activity. At the end of this period, excess of inhibitor was removed by dialysis against 0.1M-Tris/HCl, pH8, containing 100mM-KCl, 1mM-EDTA and 0.2mM-dithiothreitol for 18h at 4°C. During this period of dialysis there was no evidence for any recovery of enzyme activity, showing that 5-chloro-4-oxopentanoate produces a stable enzyme modification. Table 1 compares the rates of detritiation catalysed by both the control and inhibitor-treated enzymes, ATP, phosphate and arsenate being used as essential promoters of detritiation. The overall loss of catalytic activity was paralleled by the inability to catalyse the detritiation of pyruvate in the presence of ATP. There was a slight increase with the artificial activators, phosphate or arsenate, but only 10–14% of the control rate was obtained. This observation contrasts with the results with 2,4,6-trinitrobenzene-1-sulphonate-modified pyruvate kinase where detritiation in the presence of ATP is completely inhibited, whereas arsenate- and phosphate-stimulated detritiation are virtually unaffected (Flashner *et al.*, 1973).

Although 5-chloro-4-oxopentanoate and pyruvate do not compete with each other in the alkylation reaction, the former is an inhibitor of pyruvate detritiation in the ATP-stimulated process. The control enzyme was incubated with [3-^{3}H]pyruvate (3mM) under the conditions used in Table 1 in the presence of 0, 6, 12, 18, 24 and 30mM-5-chloro-4-oxopentanoate. This resulted in a decrease in detritiation rate of 0, 16, 26, 32.0, 39 and 48% respectively. A full kinetic analysis of this inhibition was not attempted.

Table 2. *Comparison of 5-chloro-4-oxo*[3,5-^{3}*H*]*pentanoate and* [3-^{3}*H*]*pyruvate as substrates in the detritiation assay*

The reaction conditions were as in Table 1 except that either 3mM-5-chloro-4-oxo[3,5-^{3}H]pentanoate (2.2×10^{3} d.p.m./nmol) or 5mM-[3-^{3}H]pyruvate (1.25×10^{3} d.p.m./nmol) were used as substrates.

	$10^{-4}\times{}^{3}H_2O$ (d.p.m.)	
Detritiation promoter	5-Chloro-4-oxo[3,5-^{3}H]-pentanoate	[3-^{3}H]-Pyruvate
None	1.72	1.1
ATP (2.5mM)	2.33	36.2
Phosphate (50mM)	3.82	27.0
Arsenate (50mM)	3.0	24.2

Further evidence that 5-chloro-4-oxopentanoate might bind to the active site could come from investigating whether it is itself a substrate (at pH values where the alkylation is minimal) in the detritiation assay. Table 2 compares the detritiation of 5-chloro-4-oxo[3,5-$^{3}H_4$]pentanoate with [3-^{3}H]-pyruvate. 5-Chloro-4-oxopentanoate is only a poor substrate in comparison with pyruvate; the rate of detritiation is not significantly increased by ATP but is definitely enhanced by the non-specific promoters of detritiation, i.e. phosphate or arsenate. Phosphate appears to produce the maximum rate of detritiation.

Modification of pyruvate kinase with 5-chloro-4-oxo[3,5-^{3}*H*]*pentanoate*

The experiments described so far indicate that 5-chloro-4-oxopentanoate acts as an active-site-directed inhibitor for pyruvate kinase alkylating the protein in the region of the phosphoryl overlap sites between ATP and phosphoenolpyruvate. The next step was to demonstrate that the inhibitor was bound to the enzyme in reasonable stoicheiometry with the expected number of active sites. This required modification of substantial amounts of pyruvate kinase and for this purpose it was necessary to use far higher protein concentrations than in any of the previous experiments.

Fig. 6(*a*) shows that when a stock solution of pyruvate kinase suspended in 25mM-Tris/HCl, pH7.4, containing 1mM-EDTA and 3M-$(NH_4)_2SO_4$ was added to an alkylation reaction mixture containing a fixed concentration of 5-chloro-4-oxo-pentanoate then $k_{app.}$ for inactivation decreased progressively to zero as the molar excess of inhibitor over enzyme concentration decreased below 4×10^{3}. For all practical purposes it was impossible to alkylate pyruvate kinase at concentrations above

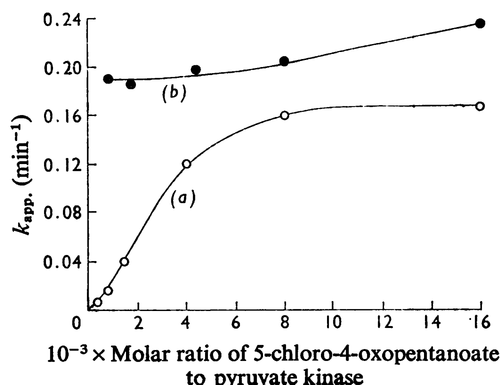


Fig. 6. *Influence of molar ratio of 5-chloro-4-oxopentanoate to pyruvate kinase on the inactivation rate constant ($k_{app.}$)*

The alkylation reaction was performed under standard conditions with 10mM-5-chloro-4-oxopentanoate and increasing concentrations of pyruvate kinase. Pyruvate kinase was added to the reaction mixture from a stock solution of enzyme in either 25mM-Tris/HCl, pH7.4, 3M-$(NH_4)_2SO_4$ and 1mM-EDTA (*a*, 28mg of protein/ml, ○) or 50mM-Tris/HCl, pH9.2, 100mM-KCl and 1mM-EDTA (*b*, 15mg of protein/ml, ●). In (*a*), pH changes due to enzyme addition were adjusted with 2M-KOH.

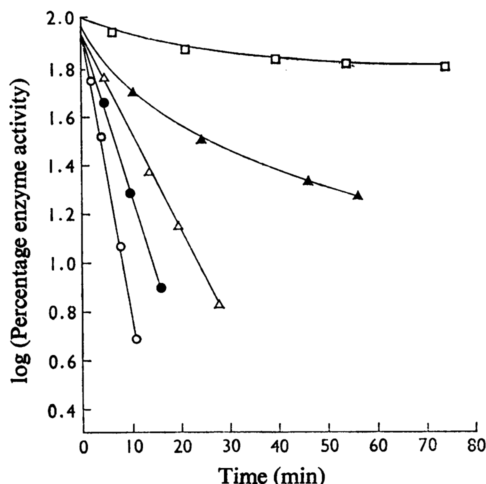


Fig. 7. *Protection of pyruvate kinase against inactivation by 5-chloro-4-oxopentanoate with $(NH_4)_2SO_4$*

Freshly dialysed pyruvate kinase was alkylated with 10mM-5-chloro-4-oxopentanoate under standard reaction conditions in the presence of several concentrations of $(NH_4)_2SO_4$; ○, none; ●, 15mM; △, 30mM; ▲, 60mM; □, 100mM.

300μg/ml by this technique. The remedy to this situation was easily found once the enzyme was dialysed into 100mM-Tris/HCl, pH9.4, containing 100mM-KCl and 1mM-EDTA. As Fig. 6(*b*) shows, with this enzyme the $k_{app.}$ for inactivation was virtually constant over the range of molar excess of inhibitor to enzyme tested. The obvious conclusion from this result is that the presence of $(NH_4)_2SO_4$ must be responsible for decreasing the $k_{app.}$ for inactivation in the earlier experiment (Fig. 6*a*). This was confirmed by incubating the dialysed enzyme with increasing concentrations of $(NH_4)_2SO_4$ (Fig. 7), which showed that $(NH_4)_2SO_4$ gave virtually complete protection against inactivation by 5-chloro-4-oxopentanoate. Further, as the concentration of $(NH_4)_2SO_4$ is increased the nature of inactivation changes from a first-order to a second-order process. In this experiment, it must be remembered that the NH_4^+ ion might bind to the essential cationic site on pyruvate kinase (Kayne, 1971). However, in our experiments, this site is saturated with 100mM-KCl, which has already been shown to give no protection against inactivation, so that it is unlikely that the NH_4^+ is protecting by virtue of binding to the enzyme. Rather, NH_4^+ ions may be protecting by virtue of their ability to react with the inhibitor.

For further alkylation experiments the enzyme was always dialysed free of $(NH_4)_2SO_4$ before use. The enzyme in this form shows pK_a and protection characteristics identical with those reported before. To determine the molar ratio for 5-chloro-4-oxopentanoate binding, 4mg of pyruvate kinase was allowed to react with 8mg of 5-chloro-4-oxo[3,5-^{3}H]-pentanoate (2.7×10^3 d.p.m./nmol) in 5ml of 50mM-Tris/HCl, pH9.4, containing 100mM-KCl and 1mM-EDTA. Over 2h, the enzyme lost 99% of its activity. The mixture was then adjusted to pH7.4 with 3M-HCl and applied to a Sephadex G-25 column. Fig. 8 (curve *a*) is an elution profile and reveals that 5-chloro-4-oxopentanoate was bound to the enzyme. As control experiments, the alkylation was performed at pH7.4 for 2h (Fig. 8, curve *b*) or at pH9.4 for 0min (Fig. 8, curve *c*) before pH7.4 treatment. Both of these treatments gave minimal inactivation of enzyme, and radioisotope incorporation was lower. Since Sephadex probably does not remove all of the non-covalently bound 5-chloro-4-oxopentanoate, the labelled enzyme was dialysed against 3×4 litres of water at 4°C over 24h. At the end of this period the inhibitor-modified enzyme contained 39.3×10^3 d.p.m./mg of protein, which corresponds to 0.85mol of inhibitor bound per mol of pyruvate kinase protomer, assuming a value of 57000 for the protomer molecular weight (Cottam *et al.*, 1969). This experiment has been repeated on ten different occasions with three different preparations of 5-chloro-4-oxo[3,5-^{3}H]-pentanoate and the values of the molar binding ratio

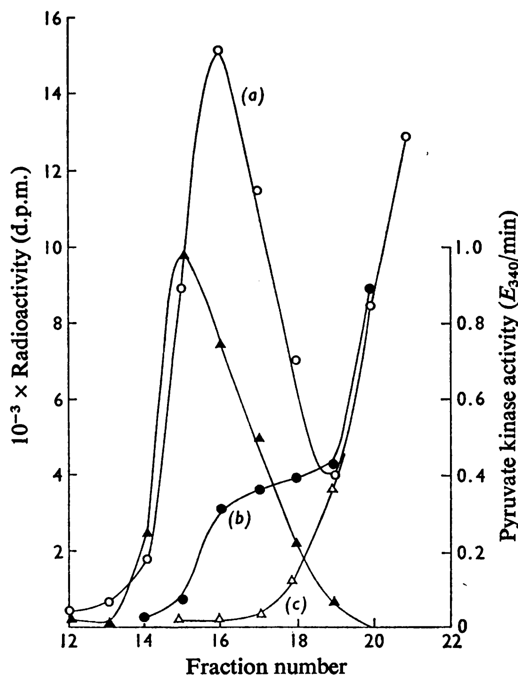


Fig. 8. *Binding 5-chloro-4-oxo*[3,5-^{3}*H*]*pentanoate to pyruvate kinase*

The result shows the distribution of 5-chloro-4-oxo-[3,5-^{3}H]pentanoate on Sephadex G-25 column (30cm × 1.5cm) chromatography in 25mM-Tris/HCl, pH7.4, following three different alkylation experiments; (*a*, ○) reaction for 2h at pH9.4; (*b*, ●) reaction for 2h at pH7.4; (*c*, △) reaction for 0min at pH9.4. The Figure also indicates the distribution of unmodified pyruvate kinase (▲).

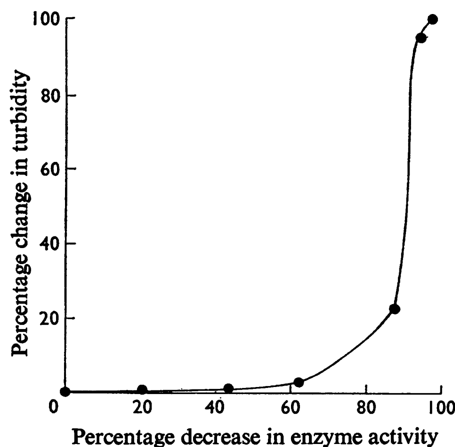


Fig. 9. *Evidence for a transition in pyruvate kinase structure during alkylation with 5-chloro-4-oxopentanoate*

Pyruvate kinase (1ml; 6.6mg of protein/ml) in 0.1M-Tris/HCl, pH9.4, containing 100mM-KCl and 1mM-EDTA was allowed to react with 10mM-5-chloro-4-oxopentanoate. Enzyme activity was assayed periodically, and E_{650} was monitored continuously. The maximum change in turbidity corresponded to a ΔE of 1.22.

range from 0.7 to 1.3. The value of 0.85 appears to represent an average value. One disadvantage of using ^{3}H-labelled 5-chloro-4-oxopentanoate, compared with a ^{14}C-labelled compound, is that ^{3}H is located in a potentially labile position. This means that during an incubation (particularly at pH9.4) the specific radioactivity of the ^{3}H-labelled compound could decline during the entire alkylation reaction and hence the molar binding ratio would be an underestimate. To check on this a number of control incubations were performed and the actual rate of ^{3}H loss was measured. Most importantly, when an alkylation reaction identical with that used in the previous experiment was set up then a negligible percentage of the total ^{3}H in 5-chloro-4-oxopentanoate was lost as ^3H_2O. Thus although the molar binding ratio may be a slight underestimate, it seems unlikely that there could be sufficient change in the specific radioactivity of the 5-chloro-4-oxo[3,5-^{3}H]-pentanoate to produce a major error.

One feature observed in the alkylation reactions using higher protein concentrations was an apparent increase in solution turbidity towards the end of the alkylation reaction. This observation was confirmed by carrying out the alkylation reaction at a higher protein concentration (Fig. 9). There was not a linear relation between enzyme inactivation and aggregation as measured by the increase in turbidity. Rather, it appeared that there was a marked increase in turbidity once the enzyme had lost 75% of its catalytic capacity. This non-linearity suggests that there may be a conformational change once the fourth protomer is modified. We feel that it is introduction of the extra negative charge on the inhibitor which is responsible for decreased enzyme solubility. Thus if enzyme that had been modified to 50% by 5-chloro-4-oxopentanoate and was quite soluble at pH9.4 was subsequently adjusted to pH7.2 with acetic acid then there was a substantial decrease in the solubility of the enzyme. Unmodified pyruvate kinase which had been exposed to pH9.4 did not show this solubility decrease until around pH5.

An alternative explanation of the increase in turbidity on 5-chloro-4-oxopentanoate modification is that once the enzyme has been modified on 75% of its subunits then subsequent alkylation is non-specific and more than one 5-chloro-4-oxopentanoate molecule is incorporated into this protomer. This possibility was eliminated by analysing the relationship between enzyme inactivation and incorporation

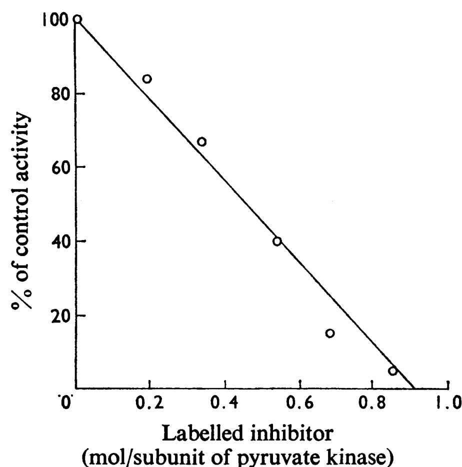


Fig. 10. *Correlation between inactivation of pyruvate kinase and incorporation of 5-chloro-4-oxo[3,5-^{3}H]pentanoate*

Pyruvate kinase (3.88mg/ml) was alkylated with 10mM-5-chloro-4-oxo[3,5-^{3}H]pentanoate (10.9×10^3 d.p.m./nmol) in 0.1M-Tris/HCl, pH9, 100mM-KCl and 1mM-EDTA. At reaction times over a 2h period small samples were removed to measure enzyme activity. Simultaneously, 770μg of protein was removed to determine bound radioactivity. Initially the sample was mixed with 0.5ml of dilute $NaBH_4$ in ice-cold water. After reduction of the carbonyl for 2min, 5mg of carrier pyruvate kinase was added followed immediately by 1ml of 10% (w/v) trichloroacetic acid. After 30min on ice, the precipitate was collected by centrifugation, washed 5 times with 5% (w/v) trichloroacetic acid and twice in acetone. The pellet was dissolved with 1ml of Hyamine hydroxide, and 0.4ml was counted for radioactivity. For 770μg of pyruvate kinase a molar binding ratio of 1.0 results in the total incorporation of 1.49×10^5 d.p.m.

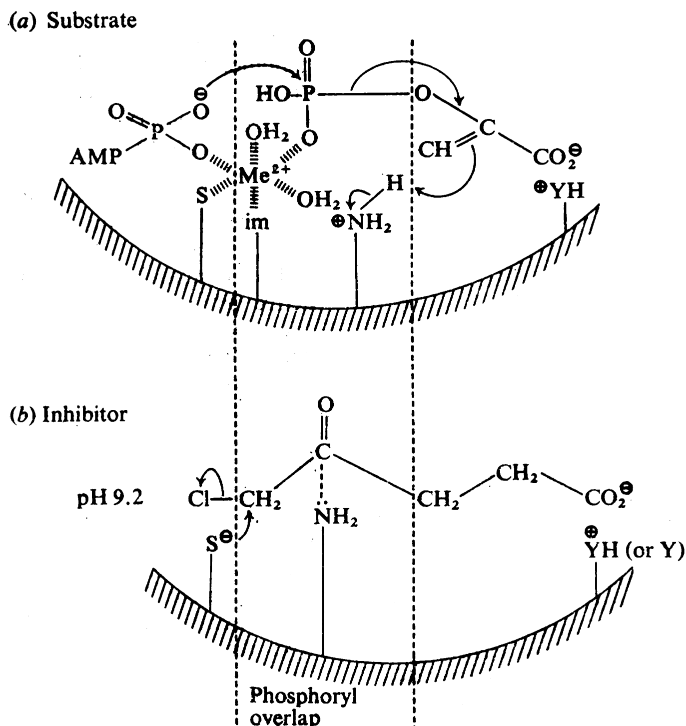
of alkylating reagent. This was found to be approximately linear (Fig. 10) down to 95% inactivation. Again this analysis indicated that the maximum inhibitor binding was 0.92mol bound per mol of pyruvate kinase protomer.

Discussion

The purpose of this work was to assess the general feasibility of using reagents of the type $Cl-CH_2-CO-[CH_2]_n-CO_2H$ for the selective alkylation of proteins. These reagents represent an alternative to the use of iodoacetate. 5-Chloro-4-oxopentanoate promotes a time-dependent and concentration-dependent irreversible inactivation of rabbit muscle pyruvate kinase. The radioactive inhibitor and the enzyme show a binding ratio of approx. 1mol of inhibitor per mol of pyruvate kinase protomer. There was a linear relationship between the incorporation of inhibitor and the inactivation of the enzyme. These results are consistent with 5-chloro-4-oxopentanoate participating in an active-site-directed alkylation of pyruvate kinase. This result confirms the general observation that the chloromethylketone functional group increases the alkylation specificity in comparison with the bromomethylketone group (10^3 times more reactive), since bromopyruvate gives multiple modification of pyruvate kinase (Flashner *et al.*, 1972).

The potential site of modification within the active site may be inferred from the protection characteristics of the various substrates and products. The active site of pyruvate kinase may be considered as two regions (Reynard *et al.*, 1961; see Scheme 1). Our experiments indicate that 5-chloro-4-oxopentanoate might alkylate a group located in the vicinity of the 'phosphoryl overlap' region. ADP and pyruvate, which do not bind within this region, provide very little protection against alkylation, whereas both ATP and phosphoenolpyruvate afford excellent protection. The complete protection by Mg^{2+} against inactivation can be explained on two grounds. First, Mg^{2+} forms a binary complex with the enzyme (Mildvan & Cohn, 1965) and combines with two negative ligands on the protein, one of which could be the cysteine thiol alkylated by 5-chloro-4-oxopentanoate (Chalkley & Bloxham, 1976). Second, examination of the co-ordination of Mg^{2+} in the ternary complexes suggests that it will also bind in the 'phosphoryl overlap' region, since either phosphoenolpyruvate or ATP act as donor ligands according to whether a substrate or product pair is present (Mildvan & Cohn, 1966; Nowak & Mildvan, 1972).

Pyruvate kinase from rabbit muscle has been alkylated by a number of reagents and there appear to be substantial differences in the protection characteristics, indicating either different modes of inhibitor binding or that the inhibitors probe different regions of the active site. The thiol groups in pyruvate kinase have been modified by *p*-chloromercuribenzoate, iodoacetamide and 5,5′-dithiobis-(2-nitrobenzoate). *p*-Chloromercuribenzoate is protected by all the components of the catalytic reaction (Mildvan & Leigh, 1964; Mildvan & Cohn, 1966) so that its action is clearly different from all other reagents. Iodoacetamide differs from 5-chloro-4-oxopentanoate in that neither phosphoenolpyruvate nor Mg^{2+} protects against inactivation (Jacobson & Black, 1971). In contrast, the main protection characteristics for both 5-chloro-4-oxopentanoate and 5,5′-dithiobis-(2-nitrobenzoate) are similar (Flashner *et al.*, 1972), i.e. protection by phosphoenolpyruvate and Mg^{2+}, but not by ADP or K^+. These results suggest that 5-chloro-4-oxopentanoate and 5,5′-dithiobis-(2-nitrobenzoate) modify the same thiol group when producing inactivation of the enzyme. Crystallographic studies



Scheme 1. *Diagrammatic representation of the orientation of functional groups at the active site of pyruvate kinase*

The scheme shows the proposed orientation of functional groups at pH7.4 in the catalytic reaction (*a*) and at pH9.2 in the alkylation reaction (*b*). The ligand formation by Me^{2+} in (*a*) is in accord with the proposals of Mildvan & Cohn (1966).

of cat muscle pyruvate kinase (Stammers & Muirhead, 1975) indicate the presence of a methylmercury-binding site (thiol group) within the active-site region.

The pK_a for the alkylation by 5-chloro-4-oxopentanoate of the cysteine group on pyruvate kinase is unusually high (pK9.2) when compared with the values reported for either iodoacetamide (pK6.8 or 7.8; Jacobson & Black, 1971) or 5,5′-dithiobis-(2-nitrobenzoate) (pK7.5; Flashner *et al.*, 1972). The high pK for 5-chloro-4-oxopentanoate inactivation is not due to its failure to react with cysteine at lower pH values. This suggests that the alkylation reaction might be controlled by a more basic group (such as ε-NH_2 of lysine) at the active site which participates in binding rather than in the displacement reaction. The initial step in binding of the inhibitor could involve interaction between the protein amino group and the inhibitor carbonyl group possibly via a Schiff base. A similar interaction has been proposed in the alkylation of 2-oxo-3-deoxy-6-phosphogluconate aldolase (Meloche, 1973). Subsequently, the chloromethyl group could be subjected to nucleophilic attack by a cysteine in the immediate vicinity of the binding site (Scheme 1). The orientation of the thiol and amino group in Scheme 1 is reasonably consistent with a simple mechanism for the catalytic reaction and their organisation is consistent with the protection data.

In summary, 5-chloro-4-oxopentanoate is a specific alkylating inhibitor of rabbit muscle pyruvate kinase. The site of inhibition is a cysteine residue which may be located near the 'phosphoryl overlap' region of the active site. The entry of the inhibitor to the active site may be controlled by a more basic group (pK9.2).

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