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USE OF METHANETHIOLATION TO INVESTIGATE THE CATALYTIC ROLE OF SULPHYDRYL GROUPS IN RABBIT SKELETAL MUSCLE PYRUVATE KINASE

DAVID P. BLOXHAM, STEPHANIE J. COGHLIN and RAM P. SHARMA

Department of Biochemistry, School of Biochemical and Physiological Sciences, University of Southampton, Southampton, S09 3TU (U.K.)

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Summary

Incubation of rabbit skeletal muscle pyruvate kinase (ATP:pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) with methyl methanethiosulphonate resulted in the time- and inhibitor concentration-dependent loss of enzyme activity. Substrates or products of the catalytic reaction prevented the loss of activity caused by methanethiolation. Their effectiveness as protecting agents was placed in the order ADP > ATP > Mg^{2+} > phospho*enol*pyruvate > pyruvate. The essential catalytic cation, K^+, had no effect on the methanethiolation reaction. [*Me*-^{3}H]Methanethiosulphonate modified all the available cysteine thiol groups which correlated to the incorporation of four SC^3H_3 groups per protomer. Four radioactive peptides were obtained on tryptic peptide mapping. When methanethiolation was carried out in the presence of Mg^{2+} alone or with Mg^{2+} and ATP together, then only three SC^3H_3 groups were incorporated into each subunit. If MgATP protected methanethiolated pyruvate kinase was reacted with iodo[2-^{3}H]acetic acid then 1.37 ± 0.2 groups per protomer were carboxymethylated. 70% of the radioactivity was located in a single peptide on tryptic peptide mapping. This peptide was isolated and contained the segment carboxymethyl cysteine (Glx, Asx, Ser) Arg. Collectively these data indicate that although all thiol groups are equally accessible to methyl methanethiosulphonate, only a single thiol group participates in the catalytic event. An additional role in the maintenance of structure for this thiol group was also shown in studied of reduction and thermal denaturation of the enzyme.

Introduction

The most readily proven catalytic roles for sulphydryl groups occur when the cysteine-SH group participates in the formation of a covalent bond with the substrate, as with glyceraldehyde 3-phosphate dehydrogenase [1], papain [2,3]

and β-ketoacyl-CoA thiolase [4]. For many enzymes the evidence for participation of a sulphydryl group is circumstantial and may be related to the fact that sulphydryl groups in proteins are amongst the most reactive amino acids. Therefore, it will be of great advantage to increase the number of reagents available for the minimal modification of thiol groups in proteins such that only small groups are introduced into the protein and the charge is not necessarily altered. Methyl methanethiosulphonate [5–7] provides a useful addition to this class of reagents since it can be used to incorporate SCH_3 groups in a reaction which may be chemically reversed by reduction [7]. An analogous reaction is the process of cyanylation [8] however this process is not chemically reversible.

As an illustration of the successful use of methyl methanethiosulphonate in minimal modification, Bloxham and Wilton [7] have shown that methanethiolation of lactate dehydrogenase at the reactive cysteine-165 resulted in an enzyme which was fully active despite the fact that this residue was previously thought to be catalytically essential [9,10]. This result corroborates crystallographic evidence on the relation of cysteine-165 to the enzyme active site [10] and sequence studies which have shown that this residue is replaced by threonine in lobster tail muscle lactate dehydrogenase [11].

In the present work, we have studied the reaction of [*Me*-^{3}H]methanethiosulphonate with rabbit muscle pyruvate kinase (ATP:pyruvate 2-*O*-phosphotransferase, EC 2.1.7.40). The modification of three thiol groups per protomer has a minor effect on enzyme activity; however, the modification of a fourth group makes the enzyme catalytically inactive. The fourth molecule of methyl methanethiosulphonate reacts at a single sequence in pyruvate kinase. This sequence appears to be important both for catalysis and maintenance of enzyme conformation.

Materials and Methods

Pyruvate kinase. Pyruvate kinase was prepared from rabbit skeletal muscle by the technique of Tietz and Ochoa [12]. The purity of each batch was confirmed by sodium dodecyl sulphate (SDS) gel electrophoresis in 7.5% gels [13]. The enzyme was routinely assayed in 25 mM Tris · HCl, pH 7.4 containing 0.1 M KCl using a lactate dehydrogenase-coupled assay system [14]. In protection experiments involving high pyruvate concentrations the enzyme was assayed by measuring the disappearance of the 230 nm absorption due to phosphoenolpyruvate [15].

For all experiments 1 ml pyruvate kinase (10 mg/ml) was dialysed for 18 h against 1 l 25 mM Tris · HCl, pH 7.4 (pH adjusted at 18°C), containing 0.1 M KCl and 1 mM EDTA at 4°C. In the small scale alkylation experiments the enzyme was diluted to 25 μg/ml in the same buffer at 0°C and suitable concentrations of methyl methanethiosulphonate and protecting ligand were added. The reaction was allowed to proceed at 0°C. 20-μl samples were removed periodically and assayed for enzymic activity.

For unmodified pyruvate kinase, the protein concentration was determined in 25 mM Tris · HCl (pH 7.4)/1 mM EDTA using an extinction coefficient at 280 nm of $30 \cdot 10^3\ l \cdot mol^{-1} \cdot cm^{-1}$ [14]. This buffer was not suitable for the

modified enzyme and 10 mM Tris · HCl (pH 7.4)/1 mm EDTA/40 mM SDS was used. The presence of the SDS restored the extinction coefficient of the modified enzyme to the same value as the control enzyme.

Methyl methanethiosulphonate. Methyl methanethiosulphonate was synthesised by H_2O_2 oxidation of dimethyl disulphide [5]. For use in methanethiolation reactions the compound was prepared as a 0.1 M solution in water.

[Me-^{3}H]Methanethiosulphonate. 12 g methane suphyryl chloride (0.11 mol) was added drop-wise to a stirred suspension of 7.2 g potassium hydrosulphide (0.1 mol) [16] in 100 ml dry tetrahydrofuran. The mixture was stirred at 50—60°C for 3 h, cooled to room temperature and filtered. The solid mass was extracted with 100 ml warm *N,N*-dimethylformamide and filtered. The filtrate was diluted with 300 ml dry diethyl ether and gave a white crystalline solid which was collected by filtration. Drying under vacuum over P_2O_5 at 20°C gave potassium methanethiosulphonate in 85% yield (melting point 202—203°C).

1.5 g potassium methanethiosulphonate (0.01 mol) was dissolved in 10 ml dry *N,N*-dimethylformamide. 25 mCi [^{3}H]methyl iodide was added and the reaction mixture was stirred at 20°C for 3 h. A further 1 g unlabelled methyl iodide (0.007 mol) was then added and the mixture was left for a further 3 h period. 50 ml water was then added and the mixture was extracted with 3 × 50 ml chloroform. The organic phase was washed with 4 × 50 ml water, dried over anhydrous Na_2SO_4 and evaporated to leave an oil. This was distilled under reduced pressure to give 250 mg [*Me*-^{3}H]methanethiosulphonate (28% yield; specific radioactivity, 2140 dpm/nmol).

Thiol modification. The thiol content of pyruvate kinase preparations was determined by adding 500 nmol 5,5′-dithiobis-(2-nitrobenzoate) in 20 μl methanol to 500 μg pyruvate kinase in 1 ml 50 mM potassium phosphate (pH 8.0)/6 M urea. Reaction was completed within 5 min and the extinction coefficient of the thionitrobenzoate released was assumed to be 13200 $l \cdot mol^{-1} \cdot cm^{-1}$ [17].

For modification of pyruvate kinase by [*Me*-^{3}H]methanethiosulphonate, the excess radioactivity was removed by crystallization from $(NH_4)_2SO_4$ solution. The modified protein was initially precipitated by the addition of 450 mg/ml enzyme-grade $(NH_4)_2SO_4$ at 0°C. After 15 min equilibration, the enzyme was collected by centrifugation at 15000 × *g* for 15 min. The enzyme was resuspended in ice-cold 25 mM Tris · HCl (pH 7.4)/0.1 M KCl/1 mM EDTA at a protein concentration of 5 mg/ml. This process was repeated a total of five times. After three crystallisations, the specific radioactivity of the protein was constant. Provided the temperature was maintained close to 0°C throughout this procedure, there was no evidence for any gross solubility change in methanethiolated pyruvate kinase preparations.

All radioisotopic counting procedures on labelled enzyme samples were carried out in toluene containing 30% (v/v) Triton X-100, 0.3% (w/v) 2,5-diphenyloxazole and 0.035% (w/v) 1,4-bis[2-(4-methyl-5-phenyloxazoyl)]-benzene using an Intertechnique ABAC-SL40 liquid scintillation counter.

Thermal denaturation of pyruvate kinase. Thermal unfolding of various species of pyruvate kinase was followed by measuring the increase in the scattering of 450 nm light using an Aminco-Bowman spectrophotofluorimeter. For

these experiments, 250 μg pyruvate kinase was incubated in 2 ml 25 mM Tris · HCl (pH 7.4)/0.1 M KCl/1 mM EDTA. The cuvette was held in a variable temperature jacket which enabled the temperature to be changed from 10 to 70°C within 5 min. The temperature in the cuvette was monitored continuously with a thermocouple. The percentage denaturation quoted in Fig. 6 is the percentage of the maximum change in light scattering.

Materials. All radioisotopic compounds were obtained from the Radiochemical Centre, Amersham, Bucks. Other materials were from the Sigma Chemical Co. Ltd., Surbiton, Surrey; Boehringer Corp. (London) Ltd., Lewes, Sussex; Koch-Light Laboratories, Colnbrook, Bucks. and B.D.H. Ltd., Poole, Dorset.

Results

Inhibition by methyl methanethiosulphonate

Reaction of rabbit muscle pyruvate kinase with methyl methanethiosulphonate resulted in a time- and inhibitor concentration-dependent inactivation of the enzyme. Plots of log $_{10}$% activity versus time were curved showing that inactivation was not pseudo first order [18]. Childs and Bardsley [19] have pointed out that the deviation from linearity is a function of the reversibility of the covalent bond formed. Certainly if a new disulphide bond is formed this could be reversed by free thiols either in solution or still unmodified on the protein.

At high concentrations of methyl methanethiosulphonate, and long reaction times (usually 2 h) then the pyruvate kinase was completely inactivated. This indicated that once the enzyme thiols were modified then the enzyme possessed no residual activity. Presumably the thiol group of pyruvate kinase plays an essential role in the catalytic process whereas for both lactate dehydrogenase and creatine kinase, which were active on thiomethylation [5,7], the role of the thiol is probably restricted to substrate binding.

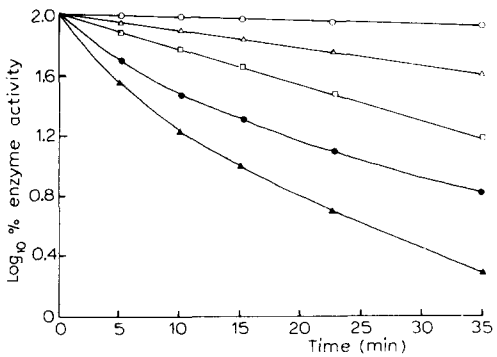


Fig. 1. Time course for the inactivation of pyruvate kinase by methyl methanethiosulphonate. The inactivation reaction was performed in 25 mM Tris · HCl (pH 7.4)/0.1 M KCl/1 mM EDTA at 0° C. The concentrations of methyl methanesulphonate used were: ▲———▲, 5 mM; ●———●, 2 mM; □———□, 0.7 mM; △———△, 0.2 mM; ○———○, 0.1 mM. There was no detectable loss of activity in the absence of inhibitor. For the assay of enzyme activity the inhibitor concentration was diluted at least 100-fold. This ensured that the inhibitor had no direct inhibitory action during the assay.

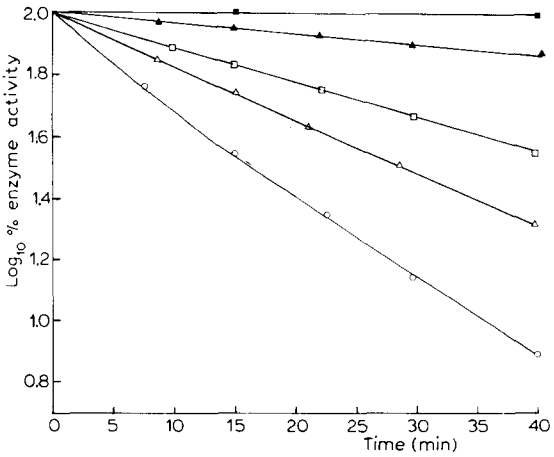


Fig. 2. Protection by ADP against the inactivation of pyruvate kinase by methyl methanethiosulphonate. Pyruvate kinase was inactivated under standard conditions in the presence of 2 mM methyl methanethiosulphonate and increasing concentrations of ADP. The concentrations of ADP were: ○———○, none; △———△, 0.05 mM; □———□, 0.1 mM; ▲———▲, 0.2 mM; ■———■, 0.5 mM.

Substrate protection

The ability of substrates to protect an enzyme against inactivation is usually taken as evidence that the inhibitor is active site directed. In a typical experiment, the loss of enzyme activity was measured in the presence of a fixed concentration of methyl methanethiosulphonate (2 mM) at increasing concentrations of a protecting ligand. Fig. 2 shows an example of the protection that was achieved in the presence of increasing concentrations of ADP. Saturating concentration of ADP provided complete protection against inactivation of the enzyme. Fig. 3 compares the protecting capability of ADP, ATP, Mg^{2+}, phos-

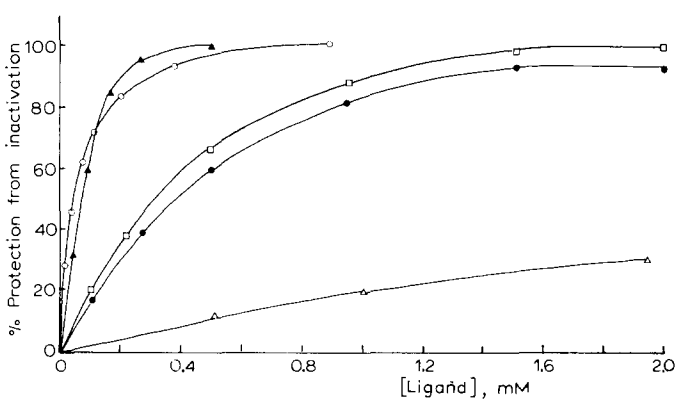


Fig. 3. Ligand protection against the inactivation of pyruvate kinase by methyl methanethiosulphonate. Protection against inactivation by 2 mM methyl methanethiosulphonate was carried out in the presence of (△———△) pyruvate, (●———●) phospho*enol*pyruvate, (□———□) Mg^{2+}, (▲———▲) ATP, or (○———○) ADP using the conditions described in Fig. 2. The protection was estimated over 40 min reaction time. The percentage protection was equal to $(k - k_p/k)100$ where k was the apparent rate constant for inactivation in the presence of inhibitor alone and k_p was the value in the presence of protecting ligand.

phoenolpyruvate and pyruvate. All five of these compounds provide detectable protection against inactivation by methyl methanethiosulphonate although it is clear that the protecting action of pyruvate was marginal. The essential cation, K^+ [20] had no effect on the rate of enzyme inactivation. For the remaining substrates the protection data was analysed by plotting the reciprocal of k_p (see Fig. 3) versus protecting ligand concentration [23]. Apparent values of dissociation constants were estimated as K_s^{ADP} 0.089 ± 0.019 mM, K_s^{ATP} 0.053 ± 0.002 mM, $K_s^{Mg^{2+}}$ 0.45 ± 0.04 mM and K_s^{PEP} 0.59 ± 0.09 mM.

Nuclear magnetic resonance experiments show that Mn^{2+} and Mg^{2+} form binary complexes directly with the enzyme [21,22] and this is confirmed by the metal ions ability to protect against inactivation. In addition to this complex, kinetic experiments substantiate the probable existence of enzyme · nucleotide · M^{2+} complexes [24,25]. We wished to see if evidence could be obtained for interaction between ATP, Mg^{2+} and enzyme in the protection experiments. To show this most effectively, single concentrations of Mg^{2+} (0.15 mM) or ATP (0.02 mM) were chosen which provided only slight protection (≈20% after 40 min reaction) against inactivation of the enzyme (Fig. 4). However, when both ligands were present simultaneously then the loss of enzyme activity was reduced (≈80% protection after 40 min reaction). The increase in protection was greater than could be obtained by the simple addition of the Mg^{2+} and ATP affects. This indicates that both Mg^{2+} and ATP interact to protect the enzyme against inactivation indicating that the two ligands bind to the enzyme in an enzyme · ATP · Mg^{2+} complex.

Binding of [Me-^{3}H]methanethiosulphonate

The reaction of thiol groups in pyruvate kinase was followed either by the disappearance of 5,5′-dithiobis-(2-nitrobenzoate)-titratable groups or by the incorporation of tritium from the labelled inhibitor. [*Me*-^{3}H]Methanethiosulphonate was synthesised so that only one methyl group was labelled and the

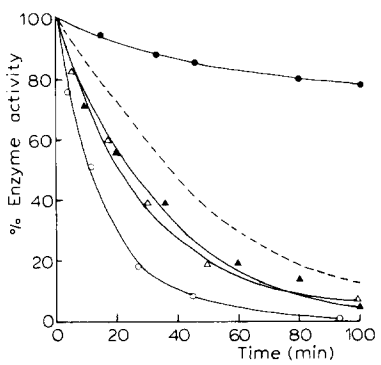


Fig. 4. Combined effect of Mg^{2+} and ATP on the inactivation of pyruvate kinase by methyl methanethiosulphonate. Inactivation of pyruvate kinase was carried out in the presence of 2 mM methyl methanethiosulphonate and the following additions; ○———○, none; △———△, 0.15 mM Mg^{2+}; ▲———▲, 0.02 mm ATP; ●———●, 0.15 mM Mg^{2+} and 0.02 mM ATP. The dashed line shows the calculated effect on inactivation by simply adding the protection when either Mg^{2+} or ATP were incubated alone.

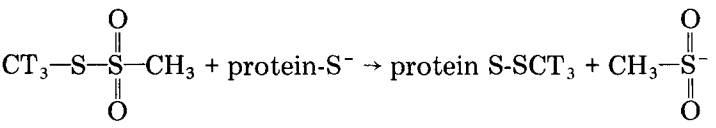
TABLE I

MODIFICATION OF THIOL GROUPS IN PYRUVATE KINASE BY [*Me*-^{3}H]METHANETHIOSULPHONATE

Pyruvate kinase (5 mg/ml) was modified at 0°C in 25 mM Tris · HCl (pH 7.4) 100 mM KCl/1 mM EDTA for 18 h. Mg^{2+} ($MgCl_2$) and ATP were included at concentrations of 5 and 0.5 mM, respectively. The concentration of [*Me*-^{3}H]methanethiosulphonate was 2 mM (2140 dpm/nmol). The enzyme was freed from excess reagent by repeated crystallisation from $(NH_4)_2SO_4$. The free thiol groups were estimated by 5,5′-dithiobis-(2-nitrobenzoate) titration. The incorporation of thio-[^{3}H]methyl groups was taken as the constant value from the third, fourth and fifth crystallisations. (The protomer molecular weight of pyruvate kinase was taken as 57 000 [35]). The number of groups modified per protomer is taken as the nearest whole number. The figure in parentheses indicates the number of experiments.

Enzyme	Specific activity (μkatals/mg protein)	Free thiol groups/protomer	Thio[^{3}H]methyl groups/protomer	Groups modified/ protomer
Control (5)	3 ± 0.15	4.4 ± 0.4	0	0
Thiomethyl pyruvate kinase (5)	0.003 ± 0.002	0.08 ± 0.01	3.6 ± 0.1	≈4
Mg^{2+}-protected thiomethyl pyruvate kinase (3)	2.1 ± 0.08	0.9 ± 0.05	2.8 ± 0.2	≈3
Mg^{2+}- and ATP-protected thiomethyl pyruvate kinase (3)	2.4 ± 0.15	0.94 ± 0.06	2.7 ± 0.15	≈3

reaction proceeded so that all the isotope was incorporated into the product, i.e.



With the preparations of pyruvate kinase used in the current work there were 4.4 ± 0.4 free thiols per monomer by 5,5′-dithiobis-(2-nitrobenzoate) titration in 6 M urea (Table I). When pyruvate kinase was reacted with [*Me*-^{3}H]methanethiosulphonate then this resulted in the disappearance of all the 5,5′-dithiobis-(2-nitrobenzoate)-titratable thiol groups and the incorporation of 3.6 ± 0.1 thiomethyl groups into the monomer. In all probability inhibition results from modification of four distinct sulphydryl groups. For the increased protein concentrations (≈0.1 mM in protomers) used in the large scale modification reactions the rate of inactivation of enzyme was much slower than in the previous experiments with dilute pyruvate kinase. However modification reactions were complete after 18 h.

The number of sites modified by methyl methanethiosulphonate was confirmed by tryptic digestion (0.2 mg trypsin/20 mg pyruvate kinase for 5 h at 37°C) of urea-denatured thiomethyl pyruvate kinase followed by peptide mapping. The two-dimensional mapping used descending chromatography for 16 h in the upper phase of butanol/acetic acid/water (4 : 1 : 5, v/v) in the first direction and high voltage electrophoresis for 1.5 h in pyridine/glacial acetic acid/water, pH 3.6 (1 : 10 : 89, v/v) in the second direction. The low pH was chosen since this gives maximum stability of the thiomethyl group during chromatography. A total of 46 spots were identified by ninhydrin. Each of these was cut out and tested for radioactivity. Of these only four spots were radioactive with chromatographic co-ordinates of 17 cm : 4.8 cm, 9.7 cm : 5.7 cm, 13.7 : 9.2 cm, 12.3 cm : 19.2 cm for directions 1 and 2, respectively. All peptides migrated in the direction of the cathode during electrophoresis.

The preceding section has shown that there is no selectivity in the reaction of methyl methanethiosulphonate with the enzyme. Specificity could be readily demonstrated by carrying out the modification reaction in the presence of protecting agents such as Mg^{2+} alone or Mg^{2+} and ATP together (Table I). In both cases there was some loss of enzyme activity, however, this was quite small in the incubation containing Mg^{2+} and ATP. Most importantly despite the exhaustive reaction with [*Me*-^{3}H]methanethiosulphonate approximately one thiol group was still intact and one less thiomethyl group (2.7 compared to 3.6) was incorporated into the protein. This result is consistent with a role for a specific cysteine sulphydryl at the enzyme active site. Note that although Mg^{2+} and ATP together enhance protection compared to Mg^{2+} alone, there is no evidence for protection of an additional sulphydryl group suggesting that both ligands interact at the same site.

The modification of a single sulphydryl residue was confirmed by a subsequent experiment involving reaction with iodo[^{3}H]acetate. In this experiment 20 mg pyruvate kinase was modified with non-radioactive 2 mM methyl methanethiosulphonate in 4 ml 25 mM Tris · HCl (pH 7.4)/0.1 M KCl/1 mM EDTA/

2 mM $MgCl_2$/0.5 mM ATP. AFter 18 h at 0°C the enzyme still retained 80% of its original activity. The protected methanethiolated enzyme was now isolated by $(NH_4)_2SO_4$ precipitation. For the second modification, 20 mg Mg^{2+}- and ATP-protected thiomethyl pyruvate kinase in 2 ml 25 mM Tris · HCl (pH 7.4)/6 M urea/1 mM EDTA was allowed to react with 1.5 mM iodo[2-^{3}H]acetic acid (potassium salt, 2400 dpm/nmol) for 24 h at 18°C. After removal of urea by dialysis the enzyme was digested with trypsin. In a typical experiment 46 nmol Mg^{2+}- and ATP-protected thiomethyl pyruvate kinase incorporated 63 nmol iodo[^{3}H]acetate corresponding to 1.37 molecules of iodoacetate incorporated per protomer of the enzyme. Generally all the values for this modification reaction were slightly higher than unity. The tryptic peptides were analysed by two-dimensional mapping using the procedure described earlier except that pyridine/glacial acetic acid/water, pH 6.5, (100 : 4 : 900, v/v) was used for the electrophoresis. Of all the ninhydrin-positive spots two were radioactive. Their co-ordinates were 21 cm : —2.5 cm, 14.2 cm : —2.0 cm (—sign indicates peptides migrated towards the anode) for chromatography and electrophoresis, respectively. The first peptide contained 70% of the total radioactivity and the second contained the remainder. This means that the main radioactive peak corresponds to about one thiol modified per protomer (1.37 × 0.7 = 0.96). Potentially this group corresponds to an active site thiol group.

Analysis of iodo[^{3}H]acetate reactive groups in Mg^{2+}- and ATP-protected thiomethyl pyruvate kinase

The major labelled peptide from experiments of the previous type was now isolated by chromatographic techniques. Initially the tryptic digest from 20 mg labelled pyruvate kinase in 25 mM NH_4HCO_3 was applied to a DEAE-cellulose column (2.5 × 25 cm) and eluted with a 500 ml linear gradient of NH_4HCO_3 (25—500 mM). Two radioactive peptides were eluted at 250 ± 10 and 310 ± 20 ml. The second peptide contained 75% of the total radioactivity and was purified further by chromatography at 45°C on a Dowex-50-2X/pyridinium column [26] using a 300 ml concave gradient between 0.2 M pyridine/acetic acid, pH 3.1, and 2 M pyridine/acetic acid, pH 5.0. A single radioactive peptide eluted from the column at 120 ± 10 ml. This was freeze dried and dissolved in water. High voltage electrophoresis in pyridine/acetic acid/water (pH 6.5) at 2.5 kV showed two ninhydrin-positive peptides migrating towards the cathode at 4.5 ± 0.2 and 9.20 ± 0.5 cm. The first peptide contained all the radioactivity and on elution was found to be pure. It was obtained in 10% overall yield based on recovery of radioactivity and amino acids. Its composition was arginine (1.0), aspartic acid (1.0), carboxymethyl-cysteine (1.0), glutamic acid (0.8) and serine (0.6). This composition is the same as that of a peptide previously isolated from reaction of an active site directed reagent, 5-chloro-4-oxopentanoic acid, with pyruvate kinase [27]. Dansylation [28] showed that the N-terminal was carboxymethyl-cysteine. Presumably the C-terminus is arginine since the peptide does not carry a strong negative charge. The reason for the discrepancy in mobility of the peptide in the purified peptide and the peptide map is not immediately explicable.

Reduction of thiomethyl pyruvate kinase

Modification of pyruvate kinase with methyl methanethiosulphonate incor-

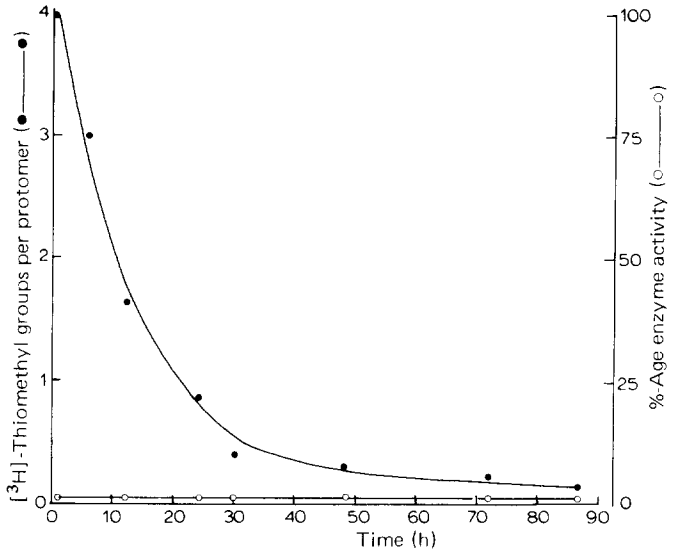


Fig. 5. Effect of dithiothreitol on the removal of thiomethyl groups from modified pyruvate kinase. [*Thiomethyl*-^{3}H]Pyruvate kinase was prepared as described in Table I. The enzyme (1 mg/ml) was reacted at 0°C in 25 mM Tris · HCl (pH 7.4)/1 mM EDTA/50 mM dithiothreitol. At appropriate times the enzyme was dialysed against 1 l 25 mM Tris · HCl (pH 7.4)/1 mM EDTA at 4°C for 24 h. The enzyme specific activity and the number of residual thiomethyl groups were then estimated.

porates -S-CH_3 groups into cysteine residues in the protein. Theoretically the disulphide bonds in the protein could be reversed by reduction and this could lead to the regeneration of the active enzyme. This type of reactivation of the modified enzyme has been demonstrated for dithiothreitol reactivation of methanethiolated lactate dehydrogenase [7]. When methanethiolated pyruvate kinase was reduced with 50 mM dithiothreitol, then quite unexpectedly there was no evidence for the reactivation of the enzyme. This could be explained either by inaccessibility of the thiomethyl groups to the reducing agent or by a methanethiolation-induced conformational change which was not reversed by reduction. To distinguish between the two possibilities, [*thiomethyl*-^{3}H]-pyruvate kinase was prepared and reduced with dithiothreitol. The thiomethyl groups were removed efficiently (Fig. 5); however there was no evidence for reactivation. This suggests that the process of thiomethylation may induce subtle structural changes in the protein which are not readily reversed by the reducing agent.

Conformational changes in pyruvate kinase

To examine potential structural changes in pyruvate kinase on methanethiolation the effect of temperature on the unfolding of pyruvate kinase was measured. For this experiment pyruvate kinase was reacted with various molar excesses of methyl methanethiosulphonate over enzyme in order to produce enzyme with 0, 1, 2 and 4 substituted thiol groups. Fig. 6 shows that whereas the unmodified enzyme showed a sharp transition with a midpoint of 61.5°C, the introduction of the thiomethyl groups produced a progressive decrease in this temperature. In a subsequent experiment methanethiolation was per-

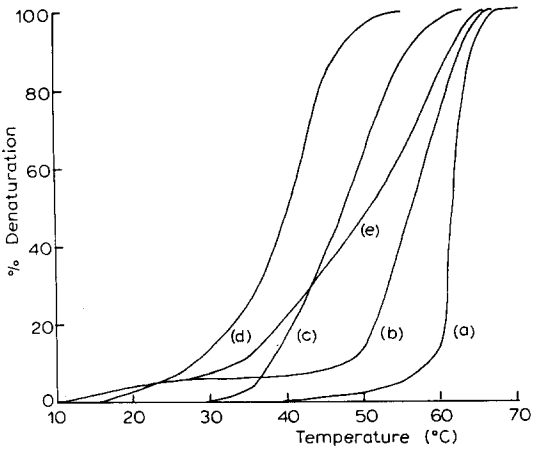


Fig. 6. Thermal denaturation of methanethiolated pyruvate kinase. For curves a, b, c and d the samples of pyruvate kinase used contained 0, 1, 2 and 4 thiomethyl groups, respectively. For curve e, Mg^{2+}- and ATP-protected thiomethyl pyruvate kinase was used.

formed in the presence of Mg^{2+} and ATP so that the active site thiol was protected. This enzyme, although it contained full catalytic activity, there was a substantial conformational change in the protein so that the mid-point in the unfolding was lowered to 47.5°C (Fig. 6, curve e) from 61.5°C in the control enzyme. It is also clear that the enzyme with the active site thiol intact is substantially different from the enzyme which was fully methanethiolated (Fig. 6, curve d). Thus it is conceivable that in addition to modifying a catalytically essential group the fourth methanethiolation reaction also produces a substantial conformational change. This could contribute to the inability to reactivate the enzyme by reduction of the methanethiolated enzyme.

Discussion

Sulphydryl groups have been clearly implicated in the catalytic mechanism of pyruvate kinase in previous studies [27,29,30]. However, there are disadvantages to the labelling procedures employed so far. 5,5′-Dithiobis-(2-nitrobenzoate) modification can be correlated to the specific modification of one group, however the modification results in the formation of an intramolecular disulphide bridge which could also result in distortion of the three-dimensional structure of the enzyme [30]. 5-Chloro-4-oxopentanoate specifically modifies one cysteine thiol group [27], however alkylation only occurs with a pK_a of 9.2 [15] which is very much greater than the optimal pH of the enzyme ($\approx$7.4). In a fresh attempt to evaluate the role of cysteine sulphydryl groups in the enzyme's catalytic activity methyl methanethiosulphonate was used to modify the enzyme. Modification of three cysteine residues did not affect enzyme activity, however, the methanethiolation of a residue in the sequence cysteine (Glx, Asx, Ser)Arg completely inactivated the enzyme. Presumably this thiol group plays an important role in catalysis either by virtue of its properties as a nucleophile or by maintenance of the correct conformational state of the protein.

Modification studies alone cannot indicate the potential location of a group within the active site; however substrate protection studies may be of value in this analysis. In the present study complete protection was provided by ADP, ATP, Mg^{2+} and phosphoenolpyruvate. The apparent dissociation constants estimated from the kinetic protection experiments were 0.089, 0.053, 0.45 and 0.59 mM for the four ligands, respectively. The values for ADP, ATP and Mg^{2+} correspond reasonably to other estimates for the formation of catalytically competent complexes involving these ligands. As an example, $K_s^{Mg^{2+}}$ estimated by competitive titration with the enzyme · Mn^{2+} complex [21] was found to be 0.38 mM and corresponds closely to the value of 0.45 mM estimated in the present work. Values for K_s^{ADP} and K_s^{ATP} are slightly lower within a factor of 2—3 of most kinetic estimates of these parameters [31—33]. The value for K_s^{PEP} differs by about an order of magnitude from most kinetic estimates of K_s^{PEP} (i.e. 0.032 → 0.075 mM) [31]. This indicates that the protection by phospho*enol*pyruvate (PEP) against inactivation by methyl methanethiosulphonate may not involve the kinetically competent enzyme · pohospho*enol*-pyruvate complex. Rather this may represent an abortive complex with the enzyme.

For the present we favour the possibility that the essential cysteine could be located close to or in co-ordination with the divalent cation binding site on the enzyme. This follows the original suggestion by Mildvan and Cohn [22] that Mn^{2+} might co-ordinate with two ligands donated by the enzyme, one of which was an imidazole group and the other a cysteine or lysine group. Recent studies [24,25] have indicated that there are probably two divalent cation binding sites at the enzyme site one of which is directly liganded to the enzyme and the other one liganded through the nucleoside di- or triphosphate. Phosphoryl groups of ATP or ADP are within the second co-ordination sphere of the enzyme-bound divalent cation. Therefore the effect of this cation must be exerted through an intervening ligand (i.e. H_2O) acting to orient or polarize the transferred phosphoryl group. In addition the enzyme · divalent cation complex may orient the protein in its correct configuration [34]. If the essential cysteine modified by methanethiolation is involved in the interaction between the metal and the enzyme then it is clear that modification of this group would result in a marked change in the properties of the enzyme by virtue of disturbing this interaction.

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