Specific inhibition of L-type pyruvate kinase by the triazine dye Procion Blue MX-R

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Incubation of the triazine dye Procion Blue MX-R with L- and M-type pyruvate kinase resulted in rapid time- and dye-concentration-dependent loss of activity. L-type pyruvate kinase was protected only by a low concentration of Mg^{2+} ; this was not the case with the M-type enzyme. Modification of the L-type form resulted in the incorporation of 1.54 ± 0.057 mol of dye/mol of enzyme subunit in the absence of Mg^{2+} , but only 0.73 ± 0.024 mol of dye/mol of enzyme subunit in the presence of Mg^{2+} . Tryptic peptide mapping of L-type pyruvate kinase modified in the presence and in the absence of Mg^{2+} further indicated that there were two sites modified in the enzyme, one of which was protected by Mg^{2+} . The pK_a of the nucleophile involved in the modification was calculated to be 7.1, implicating the possible involvement of a histidine residue. L-type enzyme was bound to Sepharose-immobilized Procion Blue MX-R specifically in the presence of Mg^{2+} , whereas binding of the M-type enzyme was Mg^{2+} -independent. The specific interaction of L-type pyruvate kinase with the dye was exploited in the large-scale purification of the enzyme and in the isolation of the phosphorylated enzyme.

Triazine dyes were introduced as textile dyes because of their ability to form stable covalent bonds with textiles under mild conditions. One such dye, Cibacron Blue F3G-A, has been used to colour high- M_r dextran to facilitate the use of the latter as a void-volume marker for gel filtration. Several proteins were found to chromatograph anomalously on gel-filtration columns if this Blue Dextran was included, owing to reversible interaction between the dye and protein (Kopperschlager *et al.*, 1971; Staal *et al.*, 1971; Ryan & Vestling, 1974). Subsequently it was shown that the Cibacron dye was specifically bound to the protein. The most likely rationale for the binding of these molecules to proteins is that the multiple aromatic and polar sulphonate groups afford considerable potential for hydrophobic and electrostatic interaction with parts of the proteins that may or may not be involved in the catalytic process (Dean & Watson, 1979).

The triazine dyes have found considerable application in enzymology (Turner, 1981; Lowe *et al.*, 1981). Most obvious is their exploitation in enzyme purification (Dean & Watson, 1979). In addition, the free dyes may react as site-specific inhibitors (Clonis & Lowe, 1980). Erythrocyte pyruvate kinase was one of the first enzymes

observed to bind to Cibacron Blue (Kopperschlager *et al.*, 1971). The L-type isoenzyme of pyruvate kinase behaves similarly (Marie & Kahn, 1977), and a Blue Sepharose step is involved in several of the recent purification schemes for this protein (Riou *et al.*, 1978; Hall *et al.*, 1979). However, the properties of covalent modification are not readily exploited for Cibacron Blue F3G-A because in this compound the triazine ring is disubstituted and the remaining chlorine group is less sensitive to nucleophilic substitution when compared with monosubstituted analogues (Lowe *et al.*, 1981). Therefore we have investigated a range of monosubstituted dyes to see whether they gave specific covalent reactions with L-type pyruvate kinase.

Materials and methods

Materials

Chemicals were obtained from BDH Chemicals (Poole, Dorset, U.K.) or Sigma Chemical Co. (Poole, Dorset, U.K.). Procion dyes were kindly given by ICI Organics Division. The catalytic subunit of cyclic AMP-dependent protein kinase was generously given by Professor P. Cohen (University of Dundee, Dundee, Scotland, U.K.).

Synthesis of Sepharose-dye conjugates

The reaction procedure of Lowe *et al.* (1980) was used to couple Sepharose 4B and Procion Blue MX-R. After 5 days, the conjugate was washed with water and then 1M-KCl until no colour was visible in the washings. After being washed with water again, the conjugate was washed with 2vol. of 1% (v/v) 2-mercaptoethanol to block any remaining chlorotriazinyl functions. After another water wash, any remaining unbound dye was stripped by washing with 2vol. of 6M-urea. After an exhaustive washing with water the conjugate could be equilibrated with the appropriate buffer. After use, the Sepharose-Procion Blue MX-R was washed with 2vol. of 6M-urea followed by 20vol. of water. The conjugate was stored in aq. 0.1% NaN₃.

Assay of pyruvate kinase

This was performed by the lactate dehydrogenase-linked spectrophotometric assay as described by Hopkirk & Bloxham (1979). One unit of pyruvate kinase catalyses the oxidation of 1 μ mol of NADH/min at 37°C.

Purification of L-type pyruvate kinase incorporating Sepharose-Procion Blue MX-R column chromatography

The details for preparation of rat liver cytosol fraction, pH precipitation, (NH₄)₂SO₄ fractionation and a first chromatography on DEAE-cellulose were as described previously (Hopkirk & Bloxham, 1979). All steps were performed at 4°C. The pooled enzyme fractions from the DEAE-cellulose column were adjusted to pH 7 by dropwise addition of cold 1M-potassium phosphate buffer, pH 7.4. This enzyme solution was adjusted to 0.1M-fructose 1,6-bisphosphate and 5mM-magnesium acetate and then dialysed overnight against 5mM-potassium phosphate, pH 7.2, containing 20% (v/v) ethylene glycol, 0.1M-dithiothreitol, 0.1M-fructose 1,6-bisphosphate and 5mM-magnesium acetate. Approx. 3000 units of enzyme were applied to a 200ml (packed volume; 8cm diam.) Sepharose-Procion Blue MX-R column previously equilibrated in dialysis buffer. Column flow (6ml/min) was interrupted for 20min and the column then washed exhaustively with buffer until A_{280} was zero. The enzyme was eluted by omission of Mg²⁺ from the washing buffer and inclusion of 1mM-EDTA. An example of this chromatographic procedure is shown in Fig. 5. High-specific-activity fractions were pooled and applied immediately to about 5ml (packed volume) of DEAE-cellulose in a 1cm-diameter column equilibrated in buffer without Mg²⁺. The pure enzyme was eluted by 0.3M-KCl in starting buffer.

Fractions (1 ml) were collected, and those with high specific activity (5–10mg of protein/ml; 270–290 units/mg of protein at 37°C) were frozen at –20°C and used as required.

The amount of enzyme applied to the Sepharose-Procion Blue MX-R column can be increased at least 4–5-fold. However, with increased loadings we have found it preferable to liberate the enzyme with buffer containing 0.25M-KCl. After removal of KCl by dialysis the enzyme may be concentrated as indicated above.

To determine protein concentrations on pure samples of enzyme an A_{280} absorbance index was determined. With stock enzyme of known A_{280} we determined both the dry residue weight after dialysis against water and the protein concentration by the method of Bradford (1976), using $A_{1cm,280}^{0.1\%} = 0.647$ for bovine serum albumin as standard ($\epsilon = 44000\text{M}^{-1}\cdot\text{cm}^{-1}$). The value obtained for L-type pyruvate kinase was $A_{1cm,280}^{0.1\%} = 0.59 \pm 0.012$, corresponding to $\epsilon = 35400\text{M}^{-1}\cdot\text{cm}^{-1}$ assuming a subunit M_r of 60000.

Enzyme inhibition

For L-type pyruvate kinase, incubations were performed at 4°C in 5mM-potassium phosphate buffer, pH 7.5, containing 2 μ M enzyme subunit, Procion dye and appropriate additions in a final volume of 250 μ l. Samples (10 μ l) were taken periodically for assay of enzyme activity. Apparent first-order rate constants were determined from plots of log(residual enzyme activity) versus time.

For the determination of reaction stoichiometry, 1mg samples of L-type pyruvate kinase were incubated with 100 μ M-Procion Blue MX-R in a reaction volume of 8.5ml. When the unprotected enzyme had lost 99% of its activity, the sample was applied to 0.5ml of DEAE-cellulose (packed volume) equilibrated with 5mM-potassium phosphate, pH 7.5. After the column had been washed with buffer, the bound protein was eluted with 0.3M-KCl and then desalted by passage through a 12cm $\times$ 1cm Sephadex G-50 column equilibrated with water. The enzyme fraction was pooled, freeze-dried and dissolved in 6M-guanidinium chloride (AristaR grade) for spectral measurements. All spectra were measured in 1cm-path-length cells.

Protein samples from modification experiments were dialysed against distilled water, freeze-dried and digested with 50 μ g of trypsin [1-chloro-4-phenyl-3-tosylamidobutan-2-one ('TPCK')-treated] in 50mM-NH₄HCO₃, pH 8, at 37°C for 4h. After treatment with 1mM-phenylmethanesulphonyl fluoride followed by freeze-drying, the tryptic peptides were dissolved in 1% (v/v) trifluoroacetic acid and mapped by analytical reverse-

phase high-pressure liquid chromatography with 30cm $\times$ 0.39cm μ Bondapak-C₁₈ column (Waters). The column was developed at a flow rate of 1.2ml/min by mixing aq. 0.12% (v/v) trifluoroacetic acid with acetonitrile containing 0.08% (v/v) trifluoroacetic acid.

Results

Inhibition of L-type pyruvate kinase by Procion Blue MX-R

Initially, a range of triazine dyes were screened for their ability to inhibit L-type pyruvate kinase. Although several compounds inhibited the enzyme at a significant rate (Table 1), the most effective was Procion Blue MX-R, and this was chosen for further study. The fact that some triazine dyes were relatively or totally ineffective at inhibiting the enzyme implies that the size and structure of the chromophore determines whether the dye can bind to the active site to inactivate the enzyme.

The irreversible inhibition of the enzyme was both time- and dye-concentration-dependent. Plots of log(residual enzyme activity) versus time were linear when the dye concentration was varied between 25 μ M to 1 mM, showing that inactivation

followed pseudo-first-order kinetics. A double-reciprocal plot of the apparent rate constant for inactivation versus inhibitor concentration gave a straight line (Kitz & Wilson, 1962), and enabled calculation of the K_d of ligand binding as $84 \pm 7 \mu$ M and the maximum rate constant for inactivation (k_r) as $2.5 \times 10^{-1} \pm 0.21 \times 10^{-1} \text{ min}^{-1}$. This analysis assumes that the inhibitor forms a Michaelis-type complex and that the formation of the covalent product is rate-limiting.

Ligand protection of L-type pyruvate kinase against inhibition

The ability of specific enzyme ligands to prevent inactivation by an irreversible inhibitor is usually taken as evidence that the inhibitor is either active-site-directed or affected by conformational changes resulting from ligand binding at the active site. When Mg^{2+} was included in the reaction mixture for inactivation of L-type pyruvate kinase then concentration-dependent protection against inhibition was observed. The apparent K_d for the enzyme- Mg^{2+} complex was evaluated (by plotting the reciprocal of the apparent first-order rate constant for inactivation against Mg^{2+} concentration) from these data to be $235 \pm 30 \mu$ M. The protection afforded by Mg^{2+} was not influenced when the metal ion was co-ordinated with nucleotides. Thus the addition of 1mM-ADP did not significantly alter the results.

A possible explanation of Mg^{2+} protection is that the bivalent cation might form an ionic interaction with the sulphonate groups on the dye (Hughes *et al.*, 1982) and the neutral complex would no longer bind to the enzyme. If this were the case then any bivalent metal ion should afford protection. However, when a number of bivalent cations were tested only those able to act as cofactors in the enzymic reaction (Mg^{2+} , Mn^{2+}) protected the enzyme from inactivation. Of the other cations tested, Ca^{2+} , Sr^{2+} and Zn^{2+} had no effect on the rate of inactivation, nor did they act as cofactors in the catalytic reaction.

The protection afforded by the bivalent metal ion was quite specific, since no other enzyme ligand (phosphoenolpyruvate, fructose 1,6-bisphosphate, pyruvate, ATP, ADP) when included alone at a concentration of 0.5mM or in combination was able to protect the L-type pyruvate kinase from inhibition.

Stoichiometry of modification of L-type pyruvate kinase

The binding of Procion Blue MX-R to the enzyme was determined spectrophotometrically. This procedure involved purifying the modified protein free from unbound dye by ion-exchange chromatography and gel filtration. During the

Table 1. Inactivation of L-type pyruvate kinase by a range of Procion dyes

L-type pyruvate kinase was inactivated by a range of Procion dyes at 100 μ M final concentration by the procedure described in the Materials and methods section.

Procion dye	Apparent rate constant for inactivation $10^2 \times k_{r(\text{app.})} (\text{min}^{-1})$
Blue MX-R	9
Yellow MX-R	7.5
Red MX-8B	7.0
Blue H3B	6.5
Orange MX-G	2.65
Blue MX-G	0.7
Red H3B	<0.024

Procion Blue MX-R

removal of free dye, there was no recovery of enzyme activity. The absorbance at 280nm and 600nm for the modified protein was then measured. The value at 280nm contains a contribution for both protein and Procion Blue MX-R absorbance. This was corrected by subtracting the dye absorbance, based on spectral measurements for free dye, which showed that the absorbance at 280nm was 2.91 times the value at 600nm. The corrected A_{280} value was used to calculate protein concentration on the basis of an experimentally determined ϵ of $35400\text{M}^{-1}\cdot\text{cm}^{-1}$ for the enzyme. Protein concentration was also measured independently by Coomassie Brilliant Blue binding (Bradford, 1976). These values were virtually identical. This implies that the spectral properties of free and bound dye are the same. This was not unexpected, since the spectrum of modified enzyme was recorded in 6M-guanidinium chloride and it was expected that the enzyme would be completely denatured.

Typical data showing the determination of binding in the presence and in the absence of Mg^{2+} are shown in Table 2. For three different enzyme preparations, the stoichiometry (mol of covalently bound dye/mol of enzyme subunit) was 0.73 ± 0.024 and 1.54 ± 0.057 in the presence and in the absence of Mg^{2+} respectively. Clearly almost twice as much dye is bound per subunit in the absence of Mg^{2+} as in its presence.

The samples from the stoichiometry experiment were dialysed against distilled water and freeze-dried. They were digested with trypsin and the resulting peptides were mapped by reverse-phase high-pressure liquid chromatography. The chromatographic effluent was monitored at 230nm to detect peptides and at 600nm to detect the covalently bound dye (Fig. 1). Essentially there were two major labelled peptide fractions when the enzyme was modified in the absence of Mg^{2+} , and only one of these remained in the presence of Mg^{2+} . The dye-labelled peptide fractions were analysed further by collecting the column effluent and directly injecting the two fractions back on to the column followed by isocratic elution at 50% (v/v) acetonitrile. The first and second fractions

contained three and four 230nm-absorbing peptides respectively. However, there were still only two 600nm-absorbing peptides. The resolution was not sufficient to allow purification of individual peptides.

Since both labelled peptide fractions are eluted at high acetonitrile concentration (approx. 50%, v/v) then they must be either very hydrophobic or fairly large in size. We have attempted to fractionate them further. However, virtually all the procedures that we have tested resulted in the loss of label from the peptide. This was particularly true when the peptide was freeze-dried from the acidic effluent off the column. Currently, this has prevented identification of the actual residue modified.

To obtain further information on the nature of the reactive groups, the pH-dependence of enzyme modification was studied. This showed a sharp midpoint pK_a of 7.1, consistent with a single reactive group being responsible for enzyme inhibition (Fig. 2).

Inhibition of M-type pyruvate kinase by Procion Blue MX-R

Numerous experiments have enforced the concept that the L- and M-type isoenzymes of pyruvate kinase are substantially different. Therefore we tested whether this was reflected in the sensitivity to dye inhibition. When incubated with Procion Blue MX-R, pure rabbit and human M-type pyruvate kinase were inhibited in a time- and dye-concentration-dependent manner. However, M-type pyruvate kinase was much less sensitive to inhibition (Fig. 2) and required both higher temperatures and greater concentrations of inhibitor. The apparent pK_a for inhibition was approx. 9.2. This value is virtually identical with the pK_a for a similar covalent reaction involving the nucleophilic displacement of halogen from 5-chloro-4-oxopentanoic acid by Cys-315 in the muscle pyruvate kinase sequence (Chalkley & Bloxham, 1976; Bloxham *et al.*, 1978; deduced from chicken gene sequence of M-type pyruvate kinase determined by Lonberg & Gilbert, 1983). None of Mg^{2+} (4mM), ADP (0.5mM), ATP

Table 2. *Stoichiometry of L-type pyruvate kinase modification by Procion Blue MX-R*

L-type pyruvate kinase (1mg) was modified in the presence and in the absence of 5mM- Mg^{2+} and purified for spectral analysis as described in the Materials and methods section. The A_{280}^{cm} (LPK) was calculated from the equation $A_{280}^{\text{cm}} (\text{LPK}) = A_{280}^{\text{cm}} - 2.91A_{600}^{\text{cm}}$. The ϵ for L-type pyruvate kinase was $35400\text{M}^{-1}\cdot\text{cm}^{-1}$ and the ϵ for bound dye was $4100\text{M}^{-1}\cdot\text{cm}^{-1}$.

Concn. of Mg^{2+} (mM)	Inhibition (%)	A_{280}^{cm}	A_{600}^{cm}	A_{280}^{cm} (LPK)	Subunit (μM)	Bound dye (μM)	Bound dye/ subunit
0	99	0.549	0.064	0.362	10.8	15.6	1.44
5	9	0.400	0.028	0.319	9.47	6.83	0.72

Fig. 1. Tryptic peptide map of Procion Blue MX-R-labelled L-type pyruvate kinase

The Figure shows part of the elution profile of trypsin-digested L-type pyruvate kinase modified with Procion Blue MX-R in the presence and in the absence of Mg^{2+} . Enzyme digestion and chromatography were performed as described in the Materials and methods section. Trace A shows A_{230} in the presence and in the absence of Mg^{2+} ; trace B shows A_{600} in the absence of Mg^{2+} ; trace C shows A_{600} in the presence of Mg^{2+} .

(0.5mM) or phosphoenolpyruvate (0.5mM) provided any significant protection of M-type pyruvate kinase inhibition.

Chromatography of isoenzymes of pyruvate kinase on Sepharose-Procion Blue MX-R

The preceding experiments provide reasonable evidence in the case of the L-type enzyme for the formation of enzyme-dye and enzyme- Mg^{2+} binary complexes. We were also interested in establishing whether an enzyme- Mg^{2+} -dye ternary complex was formed that might be analogous to an enzyme- Mg^{2+} -ADP complex. To examine this possibility we used immobilized Procion Blue MX-R and tested its ability to bind pyruvate kinase. For this procedure, the dye was coupled to Sepharose 4B and any residual functional groups

were masked by prolonged reaction with 2-mercaptoethanol. To test binding, L-type pyruvate kinase was passed through 4ml (packed volume) Sepharose-dye columns under a variety of conditions (Fig. 3). In the absence of Mg^{2+} virtually all the enzyme was found in the breakthrough volume of the column. If Mg^{2+} was included in the irrigant buffer, the L-type pyruvate kinase activity was tightly bound to the column and only a small fraction of this bound activity could be eluted with fructose 1,6-bisphosphate, phosphoenolpyruvate, Mg-ATP or Mg-ADP. However, the enzyme was efficiently eluted from the column if the Mg^{2+} was removed from the buffer and residual bivalent cation complexed with EDTA. Alternatively, the enzyme was eluted by increasing ionic strength (0.1 to 0.25M-KCl). The observation that enzyme

Fig. 2. *pH-dependence of pyruvate kinase inactivation* L-type (○) and M-type (□) pyruvate kinase were incubated at 4°C and 20°C respectively with 100 μ M-Procion Blue MX-R at different pH values, and the apparent rate constant for inactivation was measured.

Fig. 3. *Analytical chromatography of L-type pyruvate kinase on Sepharose-Procion Blue MX-R*

For both experiments, 20 units of L-type pyruvate kinase in 0.5 ml of 5 mM-potassium phosphate buffer, pH 7.2, containing 20% (v/v) ethylene glycol and 0.1 mM-dithiothreitol were applied to a 1 cm-diameter 4 ml-packed-volume column of Sepharose-immobilized dye. Column flow (1 ml/min) was then stopped for 5 min to facilitate maximum enzyme binding. In the experiment shown in (b) 5 mM-Mg²⁺ was included in the equilibrating buffer. Further changes in the equilibrating buffer were: 1, 1 mM-fructose 1,6-bisphosphate; 2, 1 mM-phosphoenolpyruvate; 3, 1 mM-ATP; 4, omission of Mg²⁺ and addition of 1 mM-EDTA.

binding is sensitive to ionic strength explains why all chromatography was performed in dilute phosphate buffers.

In contrast with the L-type enzyme, the M-form from both rabbit and human muscle was bound to Sepharose-dye both in the presence and in the absence of Mg²⁺ ions. The enzyme could not be eluted in significant yields by ATP, ADP and phosphoenolpyruvate. However, the enzyme was eluted in excellent yield (90%) by the inclusion of 0.1 M-KCl (Fig. 4). The elution was marginally better when KCl rather than NaCl was used. Thus, although the primary effect of KCl is simply elution by high salt, the preferred catalytic univalent cation is more effective.

Exploitation of Sepharose-Procion Blue MX-R columns in L-type pyruvate kinase purification

The observation that L-type pyruvate kinase was selectively bound to Sepharose-dye columns seemed ideally suited to the purpose of large-scale enzyme preparation. Affinity chromatography with Sepharose-Cibacron Blue F3G-A has been used previously in L-type pyruvate kinase preparation (Riou *et al.*, 1978). However, the process was rather complex and involved washing the column with various nucleotides before elution with parallel salt and fructose 1,6-bisphosphate gradients. Nearly all preparations of L-type pyruvate kinase are common through cytosol preparation,

Fig. 4. *Chromatography of M-type pyruvate kinase on Sepharose-Procion Blue MX-R*

Rabbit muscle pyruvate kinase (20 units) in 20 mM-Tris/HCl buffer, pH 7.4, containing 20% (v/v) ethylene glycol was applied to a 4 ml column of Sepharose-Procion Blue MX-R. The column was successively washed with buffer containing the following additions: 1, 5 mM-Mg²⁺; 2, 1 mM-MgATP; 3, 1 mM-phosphoenolpyruvate; 4, either 0.1 M-KCl (Δ) or 0.1 M-NaCl (▲).

acidification, $(\text{NH}_4)_2\text{SO}_4$ fractionation and a first DEAE-cellulose chromatography (Ljungström *et al.*, 1974; Hopkirk & Bloxham, 1979). A variety of procedures have then been exploited to obtain the final homogeneous protein with varying degrees of success. We found that this purification could be achieved by applying the enzyme prepared through the DEAE-cellulose chromatography stage (Hopkirk & Bloxham, 1979) to a large Sepharose–Procion Blue MX-R column in the presence of Mg^{2+} , washing the column until no further 280nm-absorbing material was eluted and then eluting the enzyme by removing Mg^{2+} (Fig. 5). By using this procedure the enzyme was routinely purified in 28% overall yield with 50% recovery of activity at the Sepharose–dye chromatography stage. Averaging the results for four successive preparations of the enzyme, the specific activity at 37°C was 420 ± 70 (mean $\pm$ S.E.M.) units/ A_{280} unit, corresponding to 250 units/mg of enzyme protein. The purified enzyme showed a single Coomassie Blue-stained band when analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Laemmli, 1970) with M_r 57 000–60 000 (Fig. 5). When analysed by isoelectric focusing two major bands were obtained with an additional intermediate band of lower intensity. Comparing this observation with those described by Blair *et al.* (1982) it is concluded that the two main bands represent phosphorylated and dephosphorylated forms of the enzyme.

Enzyme in intermediate stages of phosphorylation appears to be virtually absent when the enzyme is prepared through the Sepharose–dye-chromatography route.

Purification of [^{32}P]phosphorylated L-type pyruvate kinase

[^{32}P]Phosphorylated L-type pyruvate kinase was prepared by modifying the enzyme with [$\gamma\text{-}^{32}\text{P}$]ATP and the catalytic subunit of cyclic AMP-dependent protein kinase (Cohen, 1982). After reaction, the mixture was diluted 3-fold with 5 mM-magnesium acetate and applied to a Sepharose–Procion Blue MX-R column. Enzyme activity was bound to the column in exactly the same manner as the unlabelled enzyme. After the column had been washed exhaustively with Mg^{2+} -containing buffer, the phosphorylated enzyme was eluted in pure form by removal of Mg^{2+} (Fig. 6). This provides a very convenient technique for purifying the labelled protein free from [$\gamma\text{-}^{32}\text{P}$]ATP and contaminating proteins. The technique can be applied to relatively crude mixtures containing L-type pyruvate kinase with equal success.

Discussion

The affinity of various reactive chlorotriazinyl dyes for a wide range of proteins is now well documented and has been exploited in a variety of analytical and preparative procedures (Lowe *et al.*,

Fig. 5. Elution of L-type pyruvate kinase from Sepharose–Procion MX-R on a preparative scale. Approx. 3000 units of L-type pyruvate kinase from the DEAE-cellulose preparation step were purified on a 200 ml 8cm-diameter Sepharose–dye column as described in the Materials and methods section. The arrow indicates removal of Mg^{2+} . ○, Enzyme activity; ●, protein. The inserts show sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of 30 µg (1) and 60 µg (2) of enzyme purified by this route, and isoelectric focusing of the enzyme (3) in 6M-urea (O'Farrell, 1975) and isoelectric focusing (4) in the absence of ampholytes pH 6–8 (Blair *et al.*, 1982).

Fig. 6. Purification of [^{32}P]phosphorylated L-type pyruvate kinase

L-type pyruvate kinase (200 units) was phosphorylated in 5 ml of 50 mM-potassium phosphate buffer, pH 6.8, containing 10 units of the catalytic subunit of cyclic AMP-dependent protein kinase, 18 mM-magnesium acetate and 50 μCi of [$\gamma\text{-}^{32}\text{P}$]ATP (1000 $\mu\text{Ci}/\mu\text{mol}$). After 15 min, ATP was added to a final concentration of 0.1 mM. After a further 15 min, the reaction was diluted 3-fold with 20% ethylene glycol and applied to a 4 ml Sepharose-Procion Blue MX-R column. The arrow indicates omission of Mg^{2+} . Δ , Enzyme activity; $\square$, ^{32}P radioactivity; $\blacksquare$, protein. The insert shows an autoradiogram of three concentrations of labelled protein after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The M_r values ($\times 10^{-3}$) of standard proteins were 14, 18, 29, 36, 49, 57 and 68.

1981; Turner, 1981). L-type pyruvate kinase binds to Cibacron Blue F3G-A, and this has been exploited in the chromatographic purification of the enzyme on Sepharose Blue (Marie & Kahn, 1977; Riou *et al.*, 1978; Hall *et al.*, 1979). In the present work we have attempted to establish whether this affinity for chlorotriazinyl dyes could lead to specific covalent modification if appropriately reactive dyes were used. We have shown that Procion Blue MX-R does selectively modify L-type pyruvate kinase through reaction at two distinct sites. The observation of pseudo-first-order kinetics for enzyme inactivation indicates that this occurs through the initial formation of a reversible binary complex followed by the subsequent formation of a covalent complex. One reaction site is not protected by any ligand, and covalent reaction has no major effect on enzyme activity. The second site is protected by bivalent cations and reaction leads to inactivation. The exact nature of Mg^{2+} protection is open to debate. Either this could be achieved by Mg^{2+} and Procion Blue MX-R binding to the same site that contains the reactive group or, alternatively, Mg^{2+} binding could induce conformational changes that mask residues that are accessible to the reagent in the free enzyme. Conformational changes on binding Mg^{2+} to M-type pyruvate kinase are well documented (Suelter

& Melander, 1963; Kayne, 1973). Interestingly, n.m.r. studies (Meshitsaka *et al.*, 1981) showed that there was a large change in the chemical shift of one histidine residue of the muscle enzyme on binding phosphoenolpyruvate provided that Mg^{2+} and K^+ were present. There is a sharp pK_a for the inactivation of L-type pyruvate kinase of 7.1, suggesting the possible role of a histidine residue in this reaction. Potentially these two histidine residues might be equivalent in the two isoenzymes. However, generalization of results obtained with the muscle enzyme to cover the L-type enzyme must be treated with caution, since the current study has shown that the reaction of the chlorotriazinyl dye is able to distinguish rather dramatically between the two isoenzymes.

Our current model for the interaction of L-type pyruvate kinase is shown in Scheme 1. The suggestion of a binary complex between enzyme and Mg^{2+} is dependent on the observed protection against enzyme inhibition. The K_d (Mg^{2+}) ($235 \pm 30 \mu\text{M}$) determined by the ligand protection data for L-type pyruvate kinase is similar to K_d (Mg^{2+}) for M-type pyruvate kinase ($380 \pm 30 \mu\text{M}$) measured by displacement of Mn^{2+} from binary complexes (Mildvan & Cohn, 1965) and also to K_d (Mg^{2+}) ($530 \pm 30 \mu\text{M}$) determined by u.v. difference spectroscopy (Suelter & Melander,

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1963). The prediction of a binary enzyme–bivalent–cation complex is consistent with n.m.r. studies on M-type pyruvate kinase, which indicate the primary formation of a binary enzyme– Mg^{2+} complex (Mildvan & Cohn, 1965; Gupta *et al.*, 1976) and subsequent binding of the polyphosphate chain of the nucleotide in the presence of a second bivalent cation (Mildvan *et al.*, 1976; Gupta & Mildvan, 1977). The proposal of a ternary complex relies on the observation of the retention of L-type pyruvate kinase/ Mg^{2+} mixtures by Sepharose–Procion Blue MX-R columns. It seems unlikely that the Procion dye is bound to the enzyme in a similar manner to nucleotides, since we have not found any evidence that either ADP or ATP will displace the enzyme when it is immobilized on columns.

In conclusion, the work shows clearly that the original observation of affinity of pyruvate kinase for chlorotriazinyl dyes can be extended to develop a specific covalent reagent for L-type pyruvate kinase.

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