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Cysteinyl peptides labeled by dibromobutanedione in reaction with rabbit muscle pyruvate kinase

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Abstract

The bifunctional reagent 1,4-dibromobutanedione (DBBD) reacts covalently with pyruvate kinase from rabbit muscle to cause inactivation of the enzyme at a rate that is linearly dependent on the reagent concentration, giving a second order rate constant of 444 min^{-1} M^{-1}. The individual substrates phosphoenolpyruvate (with KCl), ADP, or ATP in the presence of divalent metal cation provide marked protection against inactivation suggesting that reaction occurs in the region of the active site. The limited incorporation of DBBD into pyruvate kinase was measured by reduction of the carbonyl groups of the enzyme-bound reagent using [^{3}H]$NaBH_4$. When pyruvate kinase was reacted with 120 μM DBBD at pH 7.0 for 50 min in the absence of protectants, 1.8 mol of tritium/mol of subunit was incorporated, whereas in the presence of phosphoenolpyruvate with KCl, only 1.0 mol of tritium was incorporated per mole of subunit.

Modified peptides were isolated from tryptic digests of pyruvate kinase. Reaction of enzyme in the presence of substrate (showing no activity loss) yielded a single peptide, Asn-Ile-X_1-Lys, where X_1 corresponds to Cys^{164} of the known amino acid sequence of muscle pyruvate kinase. In the absence of protectants, reaction for 10 min (when the enzyme retained substantial activity) yielded Asn-Ile-X_1-Lys as the major labeled peptide, whereas reaction for 50 min (when the enzyme was 88% inactivated) yielded predominantly Asn-Ile-X_1-Lys cross-linked to X_2-Asp-Glu-Asn-Ile-Leu-Trp-Leu-Asp-Tyr-Lys, where X_2 corresponds to Cys^{151}. Because activity loss correlates with the appearance of the cross-linked peptides but not with formation of Asn-Ile-X_1-Lys, inactivation is likely caused by the reaction leading to the cross-link between Cys^{151} and Cys^{164}. The distance between the α-carbons of these residues in the crystal structure is 15.5 Å, whereas only 12.0 Å can be spanned by the two side chains linked by a dioxobutyl group, suggesting either that pyruvate kinase undergoes a conformational change in forming the cross-link or that local rapid fluctuations in structure occur in solution to the extent of 3.5 Å in this region of pyruvate kinase.

Keywords: dibromobutanedione-labeled peptides; pyruvate kinase; rabbit muscle

Pyruvate kinase catalyzes the transfer of phosphate from PEP to ADP, the last step in glycolysis. The arrangement of substrates in the active site of the enzyme and the structural changes that occur on substrate binding and catalysis have been probed using NMR techniques and low angle X-ray scattering (Mildvan & Cohn, 1966; Gupta et al., 1976; Mildvan et al., 1976; Dunaway-Mariano et al., 1979; Nageswara Rao et al., 1979; Rosevear et al., 1987). Single crystal diffractometry at 2.6 Å resolution of the cat muscle pyruvate kinase has suggested the location of the active site and of amino acid residues that participate in substrate binding (Muirhead et al., 1986), and this information has been complemented by affinity labeling studies performed using adenine nucleotide analogues that have a chemically reactive group at various positions on the adenine ring (DeCamp et al., 1988; DeCamp & Colman, 1989; Vollmer & Colman, 1990).

In studies of the reaction of rabbit muscle pyruvate kinase with the nucleotidyl affinity labels 2-BDB-TϵA-5′-DP and 8-BDB-TA-5′-TP, loss of activity was correlated with reaction at certain residues: Cys^{164} and Tyr^{147} in the case of 2-BDB-TϵA-5′-DP (DeCamp & Colman, 1989) and predominantly Cys^{164} and Cys^{151} in the case of 8-

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Abbreviations: 2-BDB-TϵA-5′-DP, 2-[(4-bromo-2,3-dioxobutyl)thio-1,N^6-ethenoadenosine 5′-diphosphate; 8-BDB-TA-5′-TP, 8-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-triphosphate; DBBD, 1,4-dibromobutanedione; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PEP, phosphoenolpyruvate; PTH, phenylthiohydantoin; TFA, trifluoroacetic acid; DTT, dithiothreitol; and HPLC, high performance liquid chromatography.

BDB-TA-5′-TP (Vollmer & Colman, 1990). (These residues of the rabbit muscle pyruvate kinase can all be located in the amino acid sequence of the cat muscle enzyme [Muirhead et al., 1986].) The best protection against inactivation of rabbit muscle pyruvate kinase and modification of these particular residues was provided by PEP, KCl, and $MnSO_4$, indicating that reaction occurred at or near the PEP binding site. It was suggested that the specificity of the reagent was determined by the similarity between the configurations of PEP and the enol form of the chemically reactive dioxobutyl group linked to the adenine ring in 2-BDB-TεA-5′-DP and 8-BDB-TA-5′-TP. The question then arises as to whether the bromodioxobutyl group, in the absence of the nucleotidyl portion of the reagent, can itself bind to and/or react with pyruvate kinase.

This study was undertaken to elucidate the reaction of pyruvate kinase with 1,4-dibromobutanedione and to compare it with the previously described reactions of nucleotidyl analogues with pyruvate kinase (DeCamp et al., 1988; DeCamp & Colman, 1989; Vollmer & Colman, 1990). In this paper we report the kinetics of the reaction of pyruvate kinase with dibromobutanedione and the isolation and characterization of two peptides. The first peptide is seen both in the presence of protectants and in the absence of protectants at short incubation times, i.e., when complete or substantial activity is retained. A second, cross-linked peptide, whose appearance correlates with the loss of activity (and with the disappearance of the first peptide), is seen only in the absence of protectants and at longer incubation times. A preliminary version of this work has been presented (Vollmer & Colman, 1991).

Results

Inactivation of pyruvate kinase by DBBD and concentration dependence of the reaction

The incubation of rabbit muscle pyruvate kinase with 120 μM DBBD in 0.05 M HEPES buffer, pH 7.0, and 25 °C resulted in a time-dependent inactivation of the enzyme, with a rate constant for loss of activity of 0.053 min^{-1}. Control enzyme showed constant activity in the same time period.

Pyruvate kinase was incubated with DBBD at concentrations ranging from 40 to 400 μM in order to determine the dependence of the rate of inactivation on the reagent concentration. The plot of the rate constant versus the concentration of reagent shows a linear dependence on the concentration of DBBD with a second order rate constant of 444 min^{-1} M^{-1}. Note that the linear dependence on reagent concentration contrasts with the nonlinear dependence on reagent concentration of the previously studied affinity labels 8-BDB-TA-5′-DP, 8-BDB-TA-5′-TP, and 2-BDB-TεA-5-DP (DeCamp et al., 1988; DeCamp & Colman, 1989; Vollmer & Colman, 1990). The rate of the reaction of 120 μM DBBD with pyruvate kinase as measured by the loss of activity of pyruvate kinase (0.053 min^{-1}) is comparable to that of 8-BDB-TA-5′-DP (0.046 min^{-1}) at the same concentration and two to three times slower than that of 8-BDB-TA-5′-TP (0.136 min^{-1}) and 2-BDB-TεA-5′-DP (0.092 min^{-1}).

Because dibromobutanedione has more than one type of functional group capable of reacting with amino acid side chains (i.e., the diketo group and the bromoketo moiety), pyruvate kinase was incubated with butanedione in order to test whether the diketo group is sufficient for producing inactivation. Incubation of 120 μm butanedione with pyruvate kinase under the same conditions for 50 min produced no loss of activity, indicating that the bromoketo group is necessary for inactivation and that the reaction of pyruvate kinase with dibromobutanedione probably occurs at the bromoketo group by displacement of the bromide.

Effect of ligands on the inactivation of pyruvate kinase by DBBD

We investigated the effect of natural ligands on the rate of reaction of pyruvate kinase with 120 μM DBBD at concentrations of ligand that exceeded their respective dissociation constants for the enzyme–ligand complex by several fold (Mildvan & Cohn, 1966; Flashner et al., 1973). Low concentrations of divalent metal ion neither facilitate nor inhibit the reaction (Table 1, compare 2 with 5), but higher concentrations of Mn^{2+} cause marked protection against inactivation (Table 1, 6 and 7). The presence of either nucleotide ADP or ATP alone does not

Table 1. *Effect of ligands on rate of inactivation of pyruvate kinase by DBBD*[a]

Ligands added to incubation mixture	$\frac{k + \text{ligand}}{k - \text{ligand}}$
1. None	1.0
2. EDTA, 0.1 mM	1.0
3. ATP, 2 mM; EDTA, 0.1 mM	0.91
4. ADP, 2 mM; EDTA, 0.1 mM	0.87
5. $MnSO_4$, 0.050 mM	0.84
6. $MnSO_4$, 0.2 mM	0.25
7. $MnSO_4$, 2 mM	0.12
8. ATP, 1.5 mM; $MnSO_4$, 2 mM	0.10
9. ADP, 1.5 mM; $MnSO_4$, 2 mM	0.12
10. PEP, 5 mM; KCl, 100 mM; EDTA, 0.1 mM	0.42
11. PEP, 5 mM; KCl, 100 mM	0.18
12. PEP, 5 mM; KCl, 100 mM; $MnSO_4$, 0.050 mM	0.20
13. PEP, 5 mM; KCl, 100 mM; $MnSO_4$, 2 mM	0.15

[a] Pyruvate kinase (0.66 mg/mL) was incubated in 0.05 M HEPES buffer, pH 7.0, at 25 °C with 120 μM DBBD in the presence and absence of ligands.

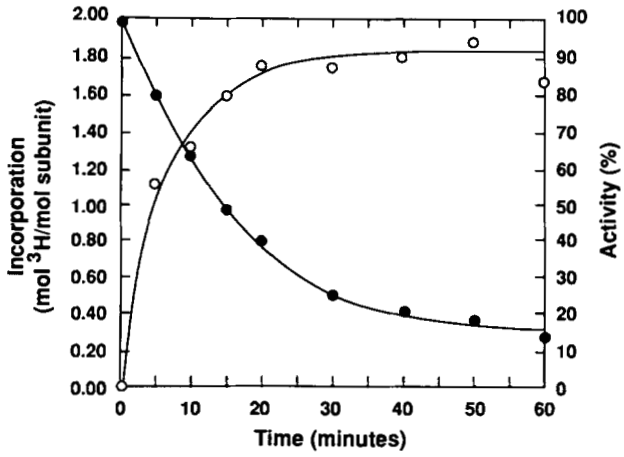


Fig. 1. Incorporation of tritium (o) into pyruvate kinase as a function of time. Pyruvate kinase (1.3 mg/mL) was incubated with 120 μM DBBD. Incorporation was determined at the indicated times, as described in the Materials and methods. The percent residual activity (•) is plotted as a function of time for comparison.

decrease the rate constant (Table 1, 3 and 4), but when divalent metal ion is also present, the decrease in k is comparable to the decrease produced by metal ion alone (Table 1, 8 and 9); these results provide no evidence for direct protection by ADP or ATP. The presence of PEP and KCl added together with EDTA (to scavenge all the free divalent metal ion) lowers the reaction rate about twofold (Table 1, 10), and low concentrations of divalent metal ion greatly strengthen this protective effect of PEP (Table 1, 11–13). These data suggest that the reaction of dibromobutanedione with the enzyme occurs in the region of the active site.

Incorporation of DBBD by pyruvate kinase

Pyruvate kinase (1.3 mg/mL) was incubated with 120 μM DBBD as described in the Materials and methods, and the incorporation of reagent into the enzyme was measured at various times during the incubation by quantitation of the tritium incorporated. As seen in Figure 1, up to 1.8 mol tritium/mol enzyme subunit was found to be enzyme bound, indicating a limited extent of reaction. Because there are two carbonyl groups in dibromobutanedione, this result may represent the incorporation of approximately 0.9 mol reagent/mol subunit.[1] It is notable that the maximum incorporation is attained at 20 min, when there is still 40% activity remaining. From 20 to 60 min the incorporation does not change although the remaining activity decreases to 15%. This result suggests that the reaction product of DBBD with enzyme may change appreciably during the course of the incubation, i.e., the initial product may not be inactive, but it may further react to form a product that is inactive. This possibility was evaluated by isolating and identifying the modified peptides formed at different times during the reaction of DBBD with pyruvate kinase.

Isolation and characterization of peptides from pyruvate kinase inactivated by reaction with DBBD

At intervals during the reaction of pyruvate kinase with 120 μM DBBD, as described in the Materials and methods, the reaction was quenched, the keto groups of the incorporated reagent were reduced with [^{3}H]$NaBH_4$, and the modified enzyme was digested with trypsin. Figure 2 illustrates a representative HPLC fractionation of tryptic digests of samples reacted for 10 min (Fig. 2A) and for 50 min (Fig. 2B,C). The distribution of radioactivity in the peptides is shown for samples derived from modified enzyme that contained 1.0 and 1.8 mol (Fig. 2A,B, respectively) of tritium/mol of subunit, and the pattern of absorbance at 220 nm is illustrated for the 50-min sample (Fig. 2C). The major radioactive peptide peaks are designated peaks I and II. It is clear that the predominant reaction product changes during the course of the reaction of pyruvate kinase with DBBD. Table 2 shows the percentages of these peaks as a function of time. Peak I is formed first; peak I then diminishes as peak II becomes apparent, with the formation of peak II correlating with the loss of activity.

Further purification of these peaks was accomplished by pooling fractions 34 and 35 (Fig. 2A) and separately pooling fractions 61 and 62 (Fig. 2B), lyophilizing each pool, and subjecting each to chromatography on a C_{18} column equilibrated with 20 mM ammonium acetate, pH 5.8, with a gradient in acetonitrile. Chromatography of fractions 34–35 gave rise to three closely spaced radioactive peaks, each of which was finally purified by re-

Table 2. *Relative percentage of peaks I and II as a function of time*[a]

Time (min)	% Inactivation	Peak I	Peak II
5	10	85	15
10	29	79	21
20	62	40	60
30	75	19	81
50	83	15	85
80	85	0	100

[1] This calculation assumes a kinetic isotope effect of 1.0. Isotope effects in the range from 0.7 to 3.0 have been reported (Pasto & Lepeska, 1976; Bailey & Colman, 1987; Ehrlich & Colman, 1987; DeCamp & Colman, 1989; Huang & Colman, 1989).

[a] Pyruvate kinase (1.3 mg/mL) was reacted with 120 μM DBBD, and, at the indicated times, aliquots were taken for tryptic digestion and fractionation by HPLC as illustrated in Figure 2 in order to obtain the percentage of peaks I and II as a function of time.

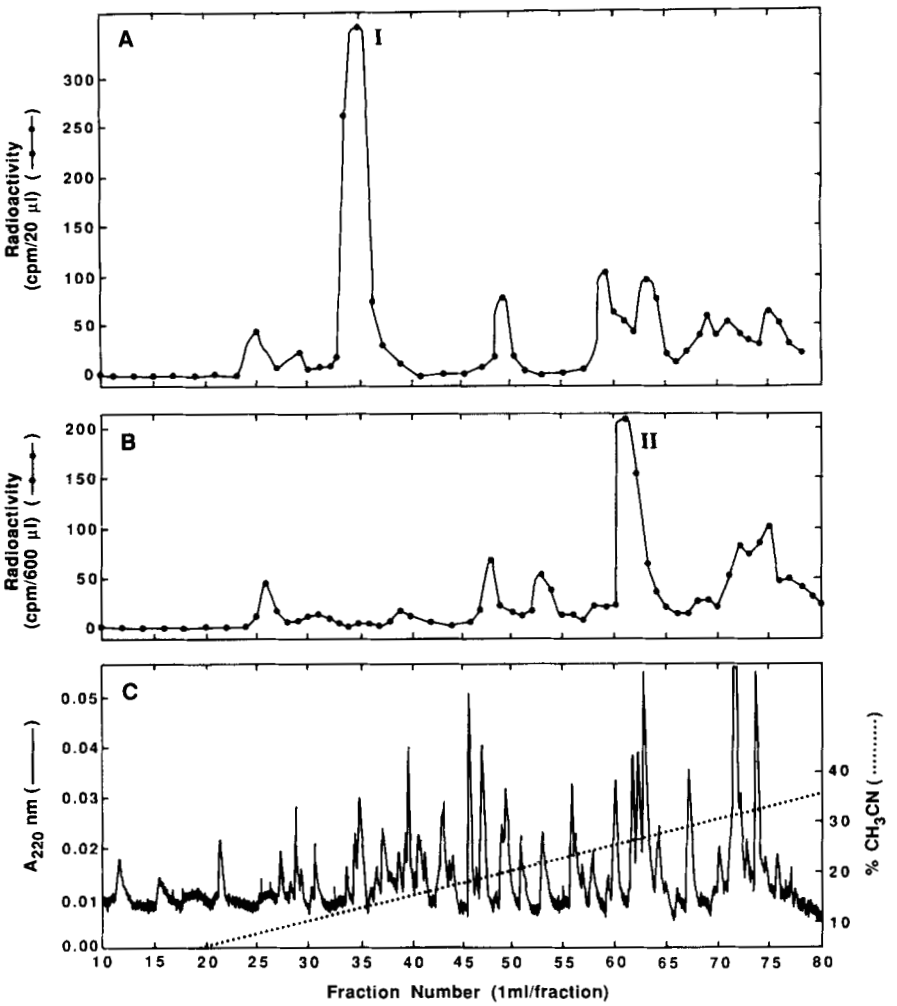


Fig. 2. Fractionation of peptides by reverse-phase HPLC. Peptides resulting from the tryptic digestion of DBBD-modified pyruvate kinase were dissolved in 0.1% TFA, applied to HPLC, and eluted using a gradient in acetonitrile (0.07% in TFA) and a flow rate of 1 mL/min. The column was eluted with 0.1% TFA for 5 min followed by a gradient to 50% acetonitrile over 100 min. **A**: Sample modified in the presence of DBBD for 10 min. **B**: Sample modified in the presence of DBBD for 50 min. **C**: A_{220} profile of run represented in B.

Table 3. *Amino acid sequence of peptides resulting from HPLC peaks*[a]

	Peptide I						Peptide II		
Cycle	Amino acid	Ia′ (pmol)	Ib′ (pmol)	Ic′ (pmol)	Ip′ (pmol)	III′ (pmol)	Amino acid	II′ (pmol)	Radioactivity (cpm)
1	Asn	18	29	168	36	143	Asn, X	187, –	3
2	Ile	20	29	166	32	168	Ile, Asp	250, 117	2
3	X	–	–	–	–	–	X, Glu	– , 156	77
4	Lys	7	6	55	21	112	Lys, Asn	124, 152	41
5							Ile	202	12
6							Leu	215	11
7							Trp	131	8
8							Leu	200	6
9							Asp	86	7
10							Tyr	154	5
11							Lys	94	3

[a] Samples denoted peak Ia′, peak Ib′, and peak Ic′ are samples derived from a digest of pyruvate kinase modified in the absence of ligands for 10 min (initial fractionation illustrated in Fig. 2A). Peak Ip′ represents a sample derived from a digest of pyruvate kinase modified in the presence of ligands for 5 min (initial fractionation illustrated in Fig. 4A). Peak III′ represents a sample resulting from fraction 23 of Figure 5B, in which pyruvate kinase reacted with DBBD for a period of 5 min was separated from excess DBBD, and the reaction was allowed to continue, giving a total time of 50 min. Peak II′ represents a sample derived from a digest of pyruvate kinase modified in the presence of DBBD for 50 min (initial fractionation represented in Fig. 2B).

chromatography on a C_{18} column equilibrated with 0.1% TFA, using a very gradual gradient in acetonitrile. Each peak yielded one peptide, identical in amino acid sequence to the others (peptides Ia′, Ib′, and Ic′ in Table 3): Asn-Ile-X-Lys, where X corresponds to Cys^{164} in the cat muscle pyruvate kinase (Muirhead et al., 1986). The multiple peaks for the same peptide may be due to the stereoisomeric products resulting from the quenching reaction with DL-DTT as well as the reduction with $NaBH_4$ (DeCamp & Colman, 1989). No radioactivity was detected in any of the PTH-amino acid fractions obtained from the peptide sequencer, indicating that the peptide–reagent bond was unstable under the sequencing conditions; similar results have been obtained with other bromodioxobutyl reagents (Vollmer & Colman, 1990).

Peak II, after further purification by HPLC, displayed a single radioactive peak corresponding to a single ultraviolet absorbance peak. The radioactive peak, upon gas phase sequencing (peptide II, Table 3), yielded two PTH-amino acids at cycles 2 and 4, indicative of a single cross-linked peptide formed from Asn-Ile-X-Lys, where X again corresponds to Cys^{164} in the cat muscle pyruvate kinase, and the peptide X-Asp-Glu-Asn-Ile-Leu-Trp-Leu-Asp-Tyr-Lys, with X corresponding to Cys^{151} in the cat muscle pyruvate kinase (Muirhead et al., 1986). Due to the absence of any amino acid in the cycle corresponding to Cys^{164} or Cys^{151}, we conclude that the cross-link between these two peptides is between these residues. In addition, radioactivity was seen in the cycle corresponding to Cys^{164}, confirming that the reagent is cross-linked to this amino acid.

Effect of PEP and KCl on the incorporation of DBBD by pyruvate kinase

PEP and KCl provide effective protection against inactivation, either in the presence or the absence of added Mn^{2+} (Table 1), and therefore the effect of these ligands on reagent incorporation was tested. The residual activity and the extent of incorporation of DBBD were measured during a 60-min reaction period at a concentration of 120 μM DBBD in the presence of 5 mM PEP and 100 mM KCl. Figure 3 shows that the reagent is rapidly incorporated during the first 5 min and that it is incorporated to the extent of approximately 1 mol ^3H/mol subunit (corresponding to little or no activity loss), after which time no further incorporation occurs. Assuming that the enzyme-bound reagent is fully reduced, i.e., 2 mol of ^3H are incorporated for each mole of enzyme-bound reagent, then 1 mol ^3H/mol subunit indicates an average of 0.5 mol reagent incorporated per mole subunit. This result suggests that the reaction occurs at, on average, 2 subunits/enzyme tetramer. (If the reagent is reduced at only one of the two keto groups, then the reaction can be assumed to be occurring with a stoichiometry of 1 mol/mol subunit.) Because the enzyme retains almost full activity, any peptides modified under these conditions are not essential for activity.

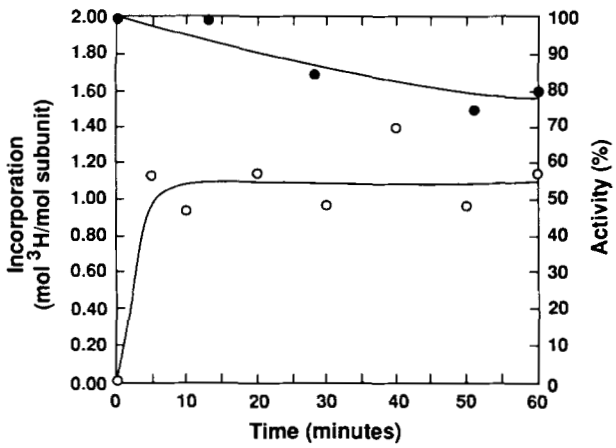


Fig. 3. Incorporation of tritium by pyruvate kinase in the presence of the protectants PEP and KCl. Pyruvate kinase (1.3 mg/mL) was incubated with 120 μM DBBD in the presence of 5 mM PEP and 100 mM KCl. The amount of tritium per mole subunit (○) was determined at the indicated time points as described in the Materials and methods. The percent residual activity (●) is plotted as a function of time for comparison.

Isolation of peptides from pyruvate kinase inactivated by reaction with DBBD in the presence of ligands

Pyruvate kinase (1.3 mg/mL) was reacted with DBBD (120 μM) at pH 7.0 with the addition of the protectant PEP with KCl. Figure 4 shows the HPLC of the tryptic digest of two samples, one that was stopped at 5 min (Fig. 4A) and a second that was stopped at 50 min (Fig. 4B). At 5 min there is only a single peak, peak I_p (which elutes in a position identical with respect to the A_{220} profile to that of peak I of the unprotected sample for the same time period). The chromatogram of the sample stopped at 50 min shows that the formation of the cross-linked peptide, seen as a small peak II_p (fraction 61), is almost completely prevented. The small amount of peak II_p present is consistent with the minor loss of activity and supports the conclusion that modification of the peptides corresponding to peak II is responsible for inactivation. The peak corresponding to the Asn-Ile-X-Lys peptide (I_p) has decreased in height, and an earlier peak, peak III_p, appears. Upon repurification of peaks I_p and III_p by HPLC in an ammonium acetate solvent system, a single peptide is obtained, and peptides I_p' and III_p' each has the sequence Asn-Ile-X-Lys (Table 3). It is probable that peak I results from reaction of Cys^{164} with dibromobutanedione to yield an initial product that still has one of the original bromoketo groups. Because the reaction of DBBD with pyruvate kinase is quenched with DTT, the peptide must also contain a molecule of DTT

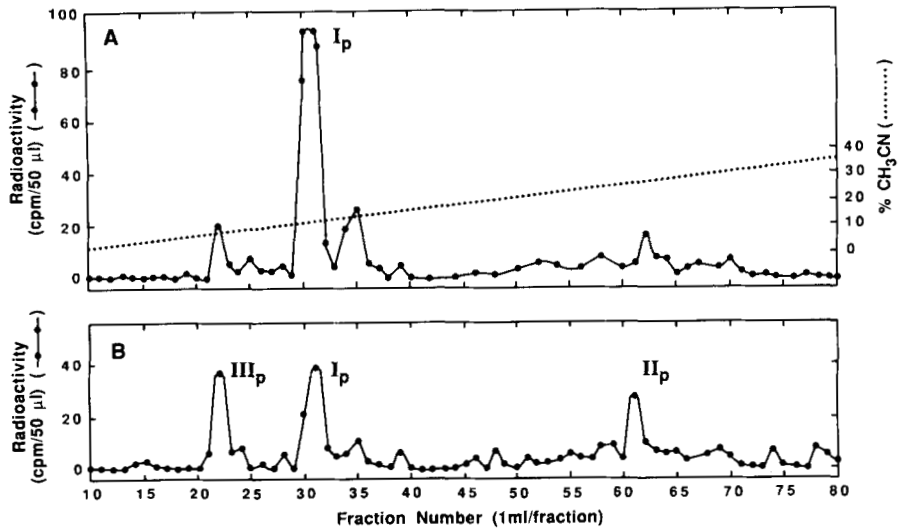


Fig. 4. Fractionation of peptides labeled in the presence of the protectants PEP and KCl. Peptides resulting from the tryptic digestion of pyruvate kinase, which had been modified by DBBD in the presence of 5 mM PEP and 100 mM KCl, were dissolved in 0.1% TFA and applied to C_{18} column using the same solvent system as described in Figure 2. **A**: Sample modified for 5 min in the presence of protectant. **B**: Sample modified for 50 min in the presence of protectant.

reacted at the residual bromoketo group. Indeed, we have noticed that when the reaction is terminated at 5 min, not with DTT but with mercaptoethanol, the resulting chromatogram shows a single radioactive peak that elutes 4 min earlier than the single radioactive peak in Figures 2A and 4A. Peak III_p probably results from the hydrolysis of the methylene bromide moiety to the corresponding alcohol. The hydrolyzed reagent will not react with the DTT used to quench the reaction and the product will be distinct, exhibiting a different migration time on HPLC, but an identical amino acid sequence.

Reaction of pyruvate kinase with DBBD when excess DBBD is removed after a 5-min reaction period

When the reaction with DBBD is carried out in the absence of ligands, peptide I, but not peptide II, is observed after 5 min of incubation time, whereas predominantly peptide II, but not peptide I, is observed after 50 min of reaction time (Fig. 2). This observation raised the possibility that the cross-linking process is an ordered process in which reaction first occurs at Cys^{164} followed by reaction at Cys^{151}. In order to investigate this possibility, we reacted pyruvate kinase with 120 µM DBBD for 5 min at pH 7.0, removed the excess DBBD by passing the reaction mixture through a Sephadex G-50–80 column equilibrated at pH 7.0 (50 mM HEPES) with 10% MeOH, and then continued the incubation of the enzyme at 25 °C for an additional 45 min before the addition of $NaBH_4$ and the preparation of tryptic peptides. Figure 5 shows the resulting HPLCs. Figure 5A shows the results from a sample in which the reaction was stopped by the addition of DTT at 5 min. It has a single peak corresponding to the peptide containing Cys^{164}. In Figure 5B, peak I has decreased, and peak II, the peptide in which Cys^{164} is cross-linked to Cys^{151}, as well as peak III (with sequence Asn-Ile-X-Lys) has appeared. This result suggests that initial reaction of DBBD at Cys^{164} can be followed by cross-linkage to Cys^{151}. The bottom panel shows the results from an aliquot of reaction mixture, which is identical to that in the center panel except for the addition at 5 min of the protectant PEP with KCl. The formation of the cross-link is substantially decreased in the presence of the substrate.

When excess dibromobutanedione is removed at 5 min from a reaction mixture containing pyruvate kinase and 120 µM DBBD at pH 7.0 (as in Fig. 5B), the enzyme continues to lose activity, but at a rate approximately 0.25 times slower than that for a sample from which excess dibromobutanedione has not been removed. This difference in the inactivation rates in the presence and in the absence of excess DBBD indicates that there is an additional mechanism operative in the formation of the cross-linked species in the presence of excess DBBD. We suggest that this mechanism most likely consists of an initial reaction at Cys^{151}, followed by cross-linkage. Because the peptide that had reacted at only Cys^{151} was never observed, this initial reaction must be succeeded by a very rapid reaction to form the cross-linked species.

Discussion

The study of the reaction of pyruvate kinase with dibromobutanedione was undertaken primarily in order to make a comparison between the reactions of pyruvate kinase with the nucleotidyl affinity labels 2-BDB-TεADP, 8-BDB-TATP, and 8-BDB-TADP, all having the bromodioxobutyl group as the reactive group, and the reactions of pyruvate kinase with the bromodioxobutyl group itself, i.e., not tethered to a nucleotide ring. The questions evident at the outset were whether DBBD would react at

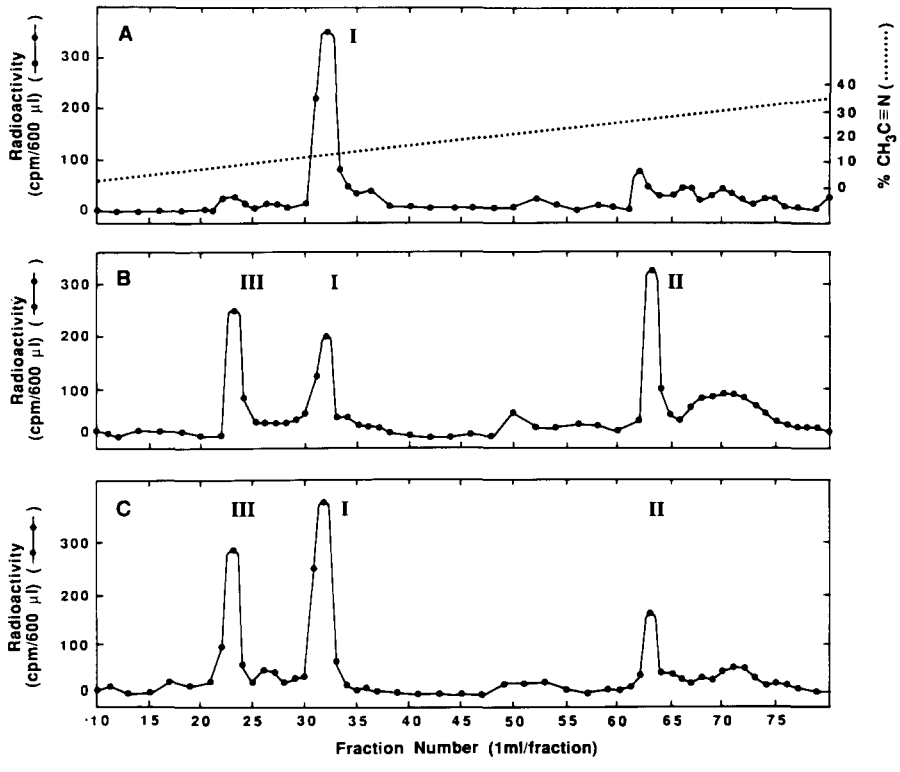


Fig. 5. Fractionation of peptides obtained by modification of pyruvate kinase for a duration of 5 min followed by removal of excess DBBD. Peptides resulting from the tryptic digestion of pyruvate kinase, which has been modified by DBBD for a period of 5 min, after which time excess DBBD was removed and dissolved in 0.1% TFA, filtered, and applied to HPLC. **A**: Sample in which the reaction was stopped at 5 min. **B**: Sample in which incubation was continued after the excess DBBD was removed. The total reaction time was 50 min. **C**: Sample in which PEP and KCl were added to the reaction mix at the time that the excess DBBD was removed, the total reaction time being 50 min.

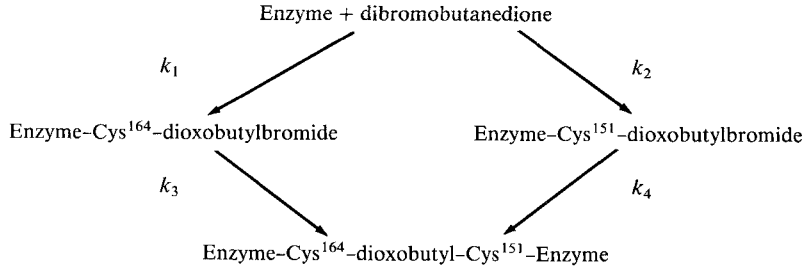
a similar rate, whether it would react at the active site, whether it might (like the nucleotidyl affinity labels) show saturation kinetics, and whether, within the active site, it would react with the same residues as those with which the nucleotidyl affinity labels reacted.

First, the inactivation rates of these various bromodioxobutyl reagents are all similar. They exhibit rates of reaction with pyruvate kinase that are all on the same order of magnitude. DBBD at a concentration of 120 μM inactivates pyruvate kinase with $k = 0.053$ min^{-1}, only slightly faster than that of 8-BDB-TADP (0.046 min^{-1}) at the same concentration and two to three times slower than that of 2-BDB-TϵADP (0.092 min^{-1}) and 8-BDB-TATP (0.136 min^{-1}).

The extent of protection by substrates against the reaction is also similar for each of these compounds. In the case of DBBD, PEP protects best against the inactivation of pyruvate kinase, indicating that reaction occurs in the region of the active site, as it does with the nucleotidyl affinity reagents bearing the bromodioxobutyl arm. The specificity for the active site of the enzyme is likely due to the similarity in structure between the substrate PEP and the dioxobutyl group, which exists predominantly in the enol form (DeCamp & Colman, 1989; Vollmer & Colman, 1990). Given that the bromodioxobutyl nucleotides react at the same functional site (that protected by PEP), it is not surprising that the particular residue that reacts with dibromobutanedione causing loss of activity, Cys^{151}, is the same residue as that which causes loss of activity upon reaction of pyruvate kinase with 8-BDB-TATP (Vollmer & Colman, 1990).

An important aspect in which dibromobutanedione differs from the nucleotidyl affinity labels in its reaction with pyruvate kinase is in the fact that DBBD does not exhibit saturation kinetics. Instead it reacts with second order kinetics, showing a linear dependence of the rate on the concentration of DBBD as measured over the range of 40–400 μM. This contrast indicates that the presence of the nucleotide moiety actually limits the reaction of the bromodioxobutyl group. It is the nucleotidyl moiety that is responsible for the binding prior to irreversible reaction.

Isolation of products as a function of time during the course of a reaction can give information concerning the intermediates of a reaction. For the reaction of DBBD with pyruvate kinase, the time course analysis of radioactive peptides by HPLC indicated that at short times a single peptide, that containing modified Cys^{164}, was isolated (with retention of most of the activity), and at longer times, the cross-linked peptide resulting from reaction at both Cys^{164} and Cys^{151} was obtained; however, at no time was the peptide containing singly modified Cys^{151} isolated. These results can be explained by reference to Scheme 1. Two alternative routes to the cross-linked Cys^{164}–dioxobutyl–Cys^{151} peptide are possible proceeding either via the k_1 plus k_3 pathway or k_2 plus k_4 pathway. In the $k_1 + k_3$ pathway, the rate-determining step appears to be k_3, whereas in the $k_2 + k_4$ pathway, the



Scheme 1.

rate-determining step is postulated to be k_2. When the initial rapid reaction of dibromobutanedione occurs at Cys^{164} (k_1), it is followed by a slower reaction (k_3) cross-linking the enzyme-bound bromodioxobutyl group to Cys^{151}; i.e., $k_1 > k_3$, as the enzyme species containing singly modified Cys^{164} accumulates. Direct evidence for the conversion of Enzyme-Cys^{164}-dioxobutylbromide to the cross-linked peptide was provided by the experiments shown in Figure 5 in which modified enzyme, freed from excess DBBD after a short reaction period, was converted to cross-linked enzyme with loss of activity. However, the observation that the inactivation rate was slower in this isolated Enzyme-Cys^{164}-dioxobutylbromide species than in the comparable enzyme sample that had not been depleted of free DBBD indicates that, in addition to the $k_1 + k_3$ route, reaction of Cys^{151} can occur directly with DBBD (k_2). In the latter case, cross-linking to Cys^{164} follows rapidly (i.e., $k_4 > k_2$), as no enzyme reacted at Cys^{151} alone ever accumulates. Because the overall kinetics of inactivation in the complete reaction mixture is directly dependent on the concentration of dibromobutanedione, the $k_2 + k_4$ pathway must be the predominant mode of reaction. Loss of activity via the $k_2 + k_4$ pathway is caused either by reaction at Cys^{151} or by cross-linking between Cys^{151} and Cys^{164}; these two events occur too close in time to be distinguished by the method of isolation of peptides.

When the preliminary reaction occurs at Cys^{164}, activity loss correlates directly with the observation of the cross-linked peptides, and inactivation can then be assumed to be caused by cross-linking of the two peptides. Examination of the crystal structure of the pyruvate kinase subunit reveals a distance of 15.5 Å between the α-carbons of residues 151 and 164 (Muirhead, pers. comm. of α-carbon coordinates). On the other hand, the C_α-CH_2-S-dioxobutyl-S-CH_2-C_α group, when fully extended (Fig. 6), can span a distance of only 12.0 Å. This measurement implies that, if the crystal structure is representative of the structure as it exists in solution, it is impossible for the bromodioxobutyl group tethered to Cys^{164} to react subsequently at Cys^{151} without some change in the structure of the enzyme. The occurrence of a conformational change in pyruvate kinase is thus suggested in forming the cross-link. The requirement for a conformational change before cross-linking can occur may be the reason that when DBBD reacts first as Cys^{164}, the second reaction, that at Cys^{151} (k_3), is significantly slower than an initial attack at Cys^{151} (k_2). Alternatively, it is possible that the 3.5 Å, which must be closed to achieve cross-linking, actually represents the ex-

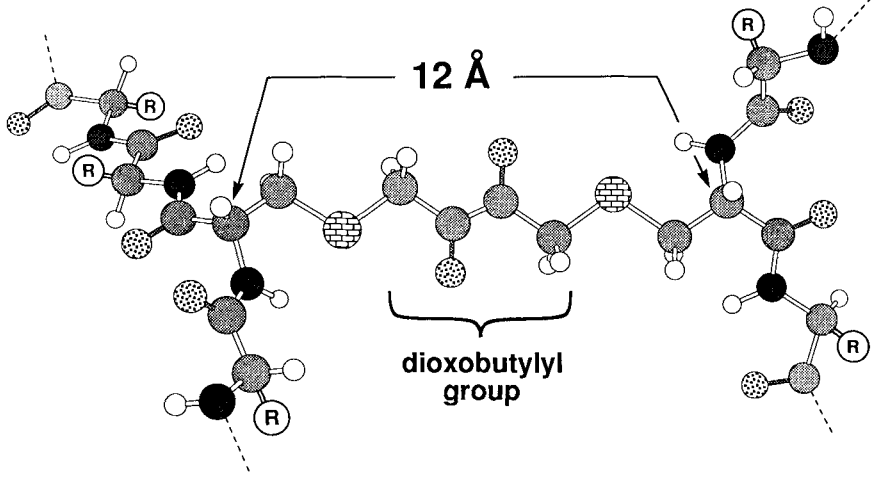


Fig. 6. Model of the product of the reaction of DBBD and two cysteine side chains. The dioxobutyl group is represented in a fully extended conformation cross-linking between two polypeptide chains. A maximum distance of 12.0 Å from the α-carbon of one polypeptide chain to the α-carbon of the second chain can be spanned by two cysteinyl side chains linked by a dioxobutyl group. The molecular modeling system Chem 3D, written by Cambridge Scientific Computing, Inc., was used to generate the structural representation.

tent of rapid local structural fluctuation occurring in solution.

The results of this study using dibromobutanedione are in accord with and strengthen our previous conclusions based on the reactions of bromodioxobutyl-nucleotides with pyruvate kinase (Vollmer & Colman, 1990). Cys^{164} is a nonessential amino acid in the vicinity of the active site, whereas modification of Cys^{151} causes loss of activity, suggesting that it is at or near the active site of muscle pyruvate kinase.

Materials and methods

Rabbit muscle pyruvate kinase was purchased from Boehringer Mannheim Biochemicals as a crystalline suspension in ammonium sulfate. The enzyme was dialyzed overnight at 4 °C against 0.05 M HEPES buffer, pH 7.0, centrifuged for 10 min at 13,000 rpm, and stored at −75 °C. The enzyme concentration was determined by using $E^{0.1\%}_{280\,nm} = 0.54$ (Bucher & Pfleiderer, 1955) and an M_r of 237,000 per tetramer (Cottam et al., 1969). Pyruvate kinase activity was measured spectrophotometrically at 340 nm by means of a coupled assay with lactate dehydrogenase. The enzymatic activity was monitored by 30 °C in 0.05 M Tris-HCl buffer, pH 7.5, containing 100 mM KCl, 10 mM $MgSO_4$, 0.5 mM PEP, 3 mM ADP, 0.25 mM NADH, and lactate dehydrogenase at a concentration of 0.1 mg/mL.

ADP, PEP, DL-DTT, and buffer salts were all obtained from Sigma Chemical Co. 1,4-Dibromobutanedione was purchased from Aldrich Chemical Co. and was recrystallized from petroleum ether before use. Lactate dehydrogenase (hog muscle) in 50% glycerol was purchased from Boehringer Mannheim Biochemicals and used without further purification. The Bio-Rad Protein Assay Dye Reagent Concentrate was from Bio-Rad Laboratories, and ultrapure guanidine hydrochloride was purchased from Schwartz/Mann. [^{3}H]$NaBH_4$ was obtained from Dupont Company (NEN) and dissolved in 0.1 N NaOH.

Determination of the kinetics of the reaction of DBBD with pyruvate kinase

Rabbit muscle pyruvate kinase (0.66 mg/mL) was incubated with a range of concentrations of DBBD at 25 °C in 0.05 M HEPES buffer, pH 7.0, for measurement of the kinetics of the reaction. Control samples were incubated under the same conditions without reagent. At timed intervals, aliquots of the reaction mixture were withdrawn, diluted with 0.05 M HEPES buffer, pH 7.0 at 25 °C, and assayed for residual pyruvate kinase activity. The rate of oxidation of NADH was measured by the loss of absorbance at 340 nm on a Cary 219 spectrophotometer. The rate of reaction of pyruvate kinase with the reagents was determined from a semilogarithmic plot of E/E_0 as a function of time, where E_0 represents the activity of the enzyme at time zero and E represents the activity at a given time. Bromodioxobutyl nucleotide derivatives have been shown to hydrolyze with the release of bromide. At pH 7 and 25 °C, the $t_{1/2}$ for hydrolysis has been shown to be about 50 min (Bailey & Colman, 1987). For this reason, the rate constants were calculated from a least-squares fit to the experimental data over the first 30 min.

Measurement of incorporation of DBBD into pyruvate kinase

The enzyme (1.3 mg/mL) was incubated with 120 μM reagent at 25 °C in 0.05 M HEPES buffer at pH 7.0. At various times a 0.5-mL aliquot of the reaction mixture was withdrawn and the reaction was stopped by the addition of DTT to a final concentration of 20 mM. Following a 1-min incubation period, 0.19 g of solid guanidine hydrochloride was added to the solution to denature the enzyme. In order to introduce a radioactive tracer by reduction of the keto groups of the enzyme-bound reagent, the modified enzyme was reduced by two additions of [^{3}H]$NaBH_4$ in 0.1 N NaOH (specific radioactivity, 2.5×10^{12} cpm/mol of hydrogen), with a 20-min interval between the additions, giving a final concentration of 3.0 mM $NaBH_4$. Twenty minutes after the second addition, the excess $NaBH_4$ was rapidly removed from the modified enzyme using the column centrifugation method of Penefsky (1979), applying the 0.5-mL sample to two successive Sephadex G-50-80 columns (5 mL) equilibrated with 0.05 M HEPES buffer, pH 7.0, and 5 M guanidine hydrochloride. The protein concentration in the eluate was determined using the Bio-Rad protein assay method based on the method of Bradford (1976), with pyruvate kinase as the protein standard. The incorporation of DBBD into pyruvate kinase was measured by the determination of the number of moles of tritium incorporated per mole of enzyme subunit.

Preparation of a proteolytic digest of enzyme modified by DBBD

Rabbit muscle pyruvate kinase (1.3 mg/mL) was incubated with 120 μM DBBD at 25 °C in 0.05 M HEPES buffer, pH 7.0. Aliquots were withdrawn during the inactivation period, diluted 8,000-fold, and assayed spectrophotometrically at 340 nm for pyruvate kinase activity by using a 0.8-mL standard assay at 30 °C. After a reaction period of 5–60 min, DTT was added to the incubation mixture to a concentration of 20 mM to stop the reaction. One minute later, solid guanidine hydrochloride was added to a concentration of about 4 M to unfold the

enzyme. Reduction with $[^3H]NaBH_4$ was accomplished as described above. Twenty minutes after the second addition of $[^3H]NaBH_4$, the free $NaBH_4$ was removed by a gel centrifugation step using Sephadex G-50-80 equilibrated with 0.05 M HEPES buffer, pH 8.0, containing 5 M guanidine hydrochloride. Iodoacetate was added to the pyruvate kinase solution in 5 M guanidine hydrochloride to a concentration of 20 mM. After 30 min at room temperature, mercaptoethanol was added to a concentration of 140 mM, followed by exhaustive dialysis against 5 mM ammonium bicarbonate. The enzyme was digested with *N*-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin, approximately 4% by weight, for 2 h at 37 °C.

Fractionation of radioactive DBBD-modified peptides by HPLC

The tryptic peptides were separated by reverse-phase HPLC on a Vydac C_{18} column (1 × 25 cm) using a Varian Model 5000 HPLC system equipped with a Varichrom absorbance monitor. Typically, the column was equilibrated with water made 0.1% in TFA (solvent A). After elution with solvent A for 5 min, a linear gradient was run to 50% solvent B (acetonitrile containing 0.07% TFA) in 110 min, followed by a linear gradient to 100% B in 20 min. In some cases, as specified in the Results, repurification of peptides was accomplished using more gradual increases in acetonitrile containing 0.07% TFA. A second solvent system was sometimes used for repurification of peptides. This involved equilibration of the column in 20 mM ammonium acetate, pH 5.8 (solvent C). The peptides were applied to the column in solvent C and were eluted using a linear gradient in solvent D (20 mM ammonium acetate in 50% acetonitrile). The flow rate was 1 mL/min. The effluent was continuously monitored for absorbance at 220 nm, and fractions of 1 mL were collected. Aliquots of fractions were mixed with 4 mL of ACS (Amersham) and were counted using a Packard Tri-Carb liquid scintillation counter, Model 1500.

Analysis of isolated peptides

Automated sequence analysis was performed on an Applied Biosystems Gas Phase Protein Sequencer, Model 470A, equipped with an online PTH analyzer, Model 120A, and computer, Model 900A. Typically, 50–1,000 pmol of peptide were analyzed.

Acknowledgments

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