nam-Evans, C. (1987) in *Tailoring Genes for Crop Improvement* (Bruening, G., Harada, J., Kosuge, T., & Hollaender, A., Eds.) pp 133–152, Plenum, New York.
Putnam-Evans, C., Harmon, A. C., & Cormier, M. J. (1986) in *Molecular and Cellular Aspects of Calcium in Plant Development* (Trewavas, A. J., Ed.) pp 99–106, Plenum, New York.
Putnam-Evans, C., Harmon, A. C., Palevitz, B. A., Fechheimer, M., & Cormier, M. J. (1989) *Cell Motil. Cytoskel. 12*, 12–22.
Sillén, L. G., & Martell, A. E. (1971) *Stability Constants of Metal–Ion Complexes*, 2nd ed., Burlington House, London.
Travis, J., Bowen, J., Tewksbury, D., Johnson, D., & Panell, R. (1976) *Biochem. J. 157*, 301–306.
Woodgett, J. R., Davison, M. T., & Cohen, P. (1983) *Eur. J. Biochem. 136*, 481–487.

Cysteinyl Peptides of Rabbit Muscle Pyruvate Kinase Labeled by the Affinity Label 8-[(4-Bromo-2,3-dioxobutyl)thio]adenosine 5′-Triphosphate†

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Received September 28, 1989; Revised Manuscript Received November 6, 1989

ABSTRACT: The affinity label 8-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-triphosphate (8-BDB-TA-5′-TP) reacts covalently with rabbit muscle pyruvate kinase, incorporating 2 mol of reagent/mol of enzyme subunit upon complete inactivation. Protection against inactivation is provided by phosphoenolpyruvate, K^+, and Mn^{2+} and only 1 mol of reagent/mol of subunit is incorporated [DeCamp, D. L., Lim, S., & Colman, R. F. (1988) *Biochemistry 27*, 7651–7658]. We have now identified the resultant modified residues. After reaction with 8-BDB-TA-5′-TP at pH 7.0, modified enzyme was incubated with [^{3}H]$NaBH_4$ to reduce the carbonyl groups of enzyme-bound 8-BDB-TA-5′-TP and to introduce a radioactive tracer into the modified residues. Following carboxymethylation and digestion with trypsin, the radioactive peptides were separated on a phenylboronate agarose column followed by reverse-phase high-performance liquid chromatography in 0.1% trifluoroacetic acid with an acetonitrile gradient. Gas-phase sequencing gave the cysteine-modified peptides Asn^{162}-Ile-Cys-Lys^{165} and Cys^{151}-Asp-Glu-Asn-Ile-Leu-Trp-Leu-Asp-Tyr-Lys^{161}, with a smaller amount of Asn^{43}-Thr-Gly-Ile-Ile-Cys-Thr-Ile-Gly-Pro-Ala-Ser-Arg^{55}. Reaction in the presence of the protectants phosphoenolpyruvate, K^+, and Mn^{2+} yielded Asn-Ile-Cys-Lys as the only labeled peptide, indicating that inactivation is caused by modification of Cys^{151} and Cys^{48}.

Pyruvate kinase catalyzes the transfer of phosphate from phosphoenolpyruvate (PEP)[1] to ADP, the last step in the glycolytic conversion of glucose to 2 molecules of pyruvate/molecule of glucose. The active site of the enzyme has been investigated through NMR techniques, which has led to predictions regarding the spatial arrangement of substrates and the structural changes that occur upon substrate binding and catalysis (Mildvan & Cohn, 1966; Gupta et al., 1976; Mildvan et al., 1976; Nageswara Rao et al., 1979; Dunaway-Mariano et al., 1979; Rosevear et al., 1987). X-ray crystallographic analysis at 2.6-Å resolution has suggested the location of the active site and of amino acid residues that participate in substrate binding (Muirhead et al., 1986). Affinity-labeling studies, which correlate loss of activity with reaction at certain residues, can yield information concerning the role and importance of regions of the active site. Such studies have identified tyrosine^{147} at the entrance to the active site as a target residue for the reactive nucleotide analogue 2-[(4-bromo-2,3-dioxobutyl)thio]-1,N^6-ethenoadenosine 5′-diphosphate (2-BDB-TεA-5′-DP) (DeCamp & Colman, 1989). Reaction of this residue with the affinity label was shown to inactivate the enzyme. Protection against inactivation of the enzyme and modification of Tyr^{147} was provided by phosphoenolpyruvate, KCl, and $MnSO_4$, indicating that reaction occurred in the region of the PEP binding site.

The related nucleotide analogues 8-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-diphosphate and 8-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-triphosphate, which feature the reactive group at a position of the purine ring different from that of 2-BDB-TεA-5′-DP, were also shown to function as affinity labels in activating rabbit muscle pyruvate kinase (DeCamp et al., 1988). As in the case of 2-BDB-TεA-5′-DP, the inactivation rate constants for 8-BDB-TA-5′-DP and 8-BDB-TA-5′-TP were markedly decreased by PEP in the presence of KCl and $MnSO_4$, whereas the coenzymes ADP and ATP were less effective in preventing inactivation. The two 8-BDB nucleotides exhibited similar kinetic characteristics in their reaction with pyruvate kinase, except that k_{max} for 8-BDB-TA-5′-TP was about 3 times greater than for 8-BDB-TA-5′-DP. After an 80-min incubation with 175 μM 8-BDB-TA-5′-TP, the enzyme was 97% inactive and 2 mol of reagent/mol of subunit were incorporated. Reaction of pyruvate kinase with 8-BDB-TA-5′-TP in the presence of the

† This research was supported by National Science Foundation Grant DMB-88-04706.

[1] Abbreviations: 2-BDB-TεA-5′-DP, 2-[(4-bromo-2,3-dioxobutyl)-thio]-1,N^6-ethenoadenosine 5′-diphosphate; 2-BOP-TεA-5′-DP, 2-[(3-bromo-2-oxopropyl)thio]-1,N^6-ethenoadenosine 5′-diphosphate; 8-BDB-TA-5′-TP, 8-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-triphosphate; 8-BOP-TA-5′-TP, 8-[(3-bromo-2-oxopropyl)thio]adenosine 5′-triphosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PEP, phosphoenolpyruvate; PTH, phenylthiohydantoin; TFA, trifluoroacetic acid.

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protectant PEP led to the incorporation of about 1 mol of reagent/mol of subunit with only a 20% loss in activity.

In this paper we report the isolation and characterization of one peptide that is modified both in the presence and in the absence of substrate and two critical peptides that are modified only in the absence of substrate. The differences between the active-site residues tagged by use of 8-BDB-TA-5′-TP and 2-BDB-TεA-5′-DP are expected to reflect the difference in position of the reactive group with respect to the adenine ring. A preliminary version of this work has been presented (Vollmer & Colman, 1989).

Experimental Procedures

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 $E^{0.1\%}_{280nm}$ = 0.54 (Bucher & Pfleiderer, 1955) and a 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 at 30 °C in 0.05 M Tris-HCl buffer, pH 7.5, containing 100 mM KCl, 10 mM $MgSO_4$, 0.05 mM phosphoenolpyruvate, 3 mM ADP, 0.25 mM NADH, and lactate dehydrogenase at a concentration of 0.1 mg/mL. 8-BDB-TA-5′-TP was synthesized as described previously (DeCamp et al., 1988). 8-[(3-Bromo-2-oxopropyl)thio]-adenosine 5′-triphosphate (8-BOP-TA-5′-TP) was synthesized, and a rate constant for inactivation of pyruvate kinase was measured by DeCamp (1988). The synthesis was initiated by preparation of the pyridinium salt of 8-TA-5′-TP in accordance with the synthesis of 8-BDB-TA-5′-TP; coupling of 1,3-dibromo-2-propanone with 8-TA-5′-TP was accomplished by using a method similar to that described for 2-[(3-bromo-2-oxopropyl)thio]-1,N^6-ethenoadenosine 5′-diphosphate (2-BOP-TεA-5′-DP) (DeCamp & Colman, 1989). [^{3}H]$NaBH_4$, dissolved in 0.1 N NaOH, was obtained from New England Nuclear Corp. Phenylboronate agarose (matrex gel PBA-30) was purchased from Amicon Corp.

Molecular modeling on the analogues was carried out by using the program Chem3D (Cambridge Scientific Corp.). In the case of pyruvate kinase, molecular modeling involved the use of the atomic coordinates provided by Prof. Hilary Muirhead (personal communication) on a Silicon Graphics IRIS 3030 system by application of the program QUANTA from Polygen.

Preparation of Enzyme Modified by 8-BDB-TA-5′-TP. Rabbit muscle pyruvate kinase (1.0 mg/mL) was incubated with 175 μM 8-BDB-TA-5′-TP at 25 °C in 0.05 M HEPES buffer, pH 7.0. Aliquots were withdrawn during the inactivation period, diluted 8000-fold, and assayed spectrophotometrically at 340 nm for pyruvate kinase activity by using an 0.80-mL standard assay at 30 °C. After 80 min of reaction of enzyme with 8-BDB-TA-5′-TP, dithiothreitol 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 4 M and the unreacted 8-BDB-TA-5′-TP was removed by using the gel centrifugation procedure of Penefsky (1979). Each 0.5 mL of reaction mixture was applied to a 5-mL column of Sephadex G-50–80 equilibrated with 0.05 M HEPES buffer, pH 7.0, containing 5 M guanidine hydrochloride.

Reduction of 8-BDB-TA-5′-TP-Modified Enzyme by [^{3}H]$NaBH_4$. To introduce a radioactive tracer into 8-BDB-TA-5′-TP-modified enzyme, the modified enzyme was treated with [^{3}H]$NaBH_4$ to reduce the keto groups of the reagent which was covalently linked to the protein. The modified enzyme was reduced by two additions of [^{3}H]$NaBH_4$ in 0.1 M NaOH (specific radioactivity, 2.5 × 10^{12} cpm/mol of hydrogen), with a 20-min interval between additions, giving a final concentration of 3 mM $NaBH_4$. Twenty minutes after the second addition, the free sodium borohydride 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.

Carboxymethylation and Proteolytic Digestion of Modified Enzyme. Iodoacetic acid 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. This was followed by exhaustive dialysis against 5 mM ammonium bicarbonate and a final dialysis against 20 mM potassium phosphate, pH 8.0. The enzyme was digested with *N*-tosyl-L-phenylalanine chloromethyl ketone treated trypsin, approximately 4% by weight, for 2 h at 37 °C. The samples were then applied to the phenylboronate agarose column.

Separation of Radioactive 8-BDB-TA-5′-TP Peptides by Chromatography on Phenylboronate Agarose. The nucleotidyl peptides were purified on a phenylboronate agarose (PBA-30) column (1 × 27 cm) equilibrated with 50 mM potassium phosphate, pH 8.0. The unmodified peptides were eluted in 50 mM potassium phosphate; a water wash then eluted the nucleotidyl peptides. Column fractions were monitored for 220-nm absorbance and for radioactivity. Appropriate fractions were pooled and lyophilized.

Fractionation of Radioactive 8-BDB-TA-5′-TP-Modified Peptides by High-Performance Liquid Chromatography (HPLC). The nucleotidyl 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. The column was equilibrated with water made 0.1% in trifluoroacetic acid (solvent A). After elution with solvent A for 5 min, a linear gradient was run to 21% solvent B (acetonitrile containing 0.07% TFA) in 48 min, followed by a linear gradient to 29% solvent B in 120 min. 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 5 mL of ACS (Amersham) and were counted by using Packard Tri-Carb liquid scintillation counter, Model 1500.

Analysis of Isolated Peptides. Automated sequence analysis was performed on an Applied Biosystems gas-phase protein (peptide) sequencer, Model 470, equipped with an online phenylthiohydantoin analyzer, Model 120, and computer, Model 900A. Typically 100–1000 pmol of peptide was analyzed.

Results

Isolation of Peptides from Pyruvate Kinase Inactivated by Reaction with 8-BDB-TA-5′-TP. Rabbit muscle pyruvate kinase (1.0 mg/mL) was 97% inactivated by incubation for 80 min with 175 μM 8-BDB-TA-5′-TP at pH 7.0 and 25 °C with 2 mol of reagent incorporated/mol of subunit. Following reduction of the keto groups with [^{3}H]$NaBH_4$ and removal of noncovalently bound reagent, the modified enzyme was carboxymethylated and then digested with trypsin as described under Experimental Procedures. The tryptic digest was applied to a phenylboronate agarose column (PBA-30), which forms a reversible complex with the *cis*-hydroxyl groups of 8-BDB-TA-5′-TP. Nucleotidyl peptides are selectively adsorbed to

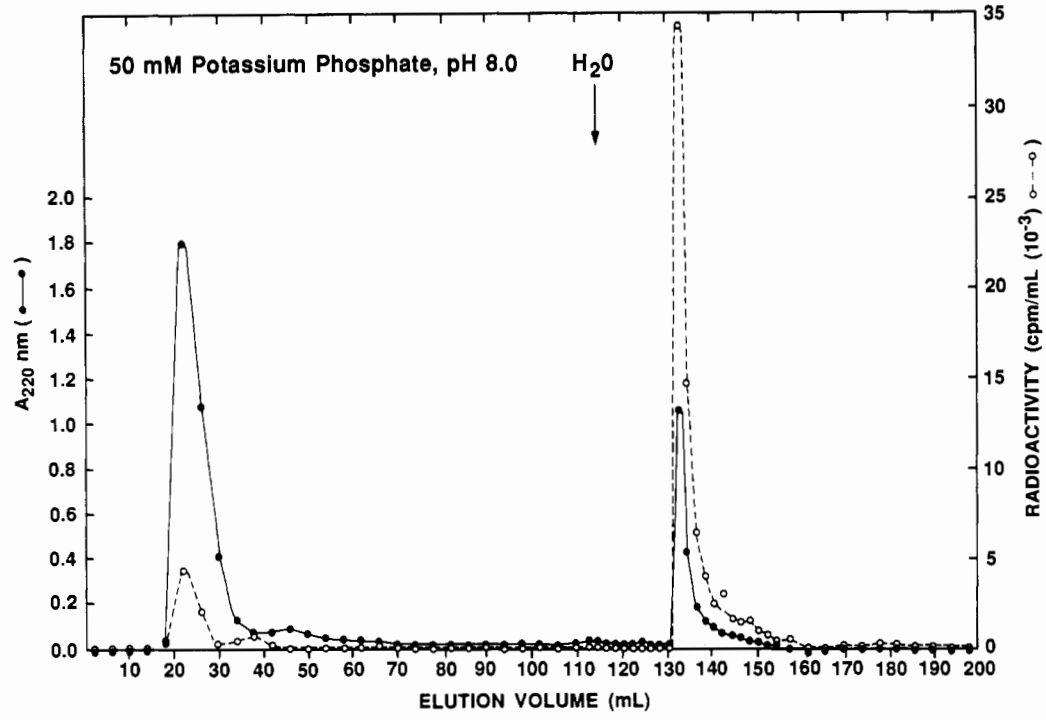

FIGURE 1: Separation of nucleotidyl peptides on phenylboronate agarose. Peptides resulting from the tryptic digestion of 3 mg of 8-BDB-TA-5′-TP-modified pyruvate kinase were made 50 mM in potassium phosphate, pH 8.0, and applied to a PBA-30 column. The column was washed with the potassium phosphate buffer, pH 8.0, followed by elution of the bound peptides with water as indicated by the arrow. Fractions were monitored for A_{220nm} (●) and for radioactivity (○). The material eluting between 134 and 140 mL was pooled and lyophilized.

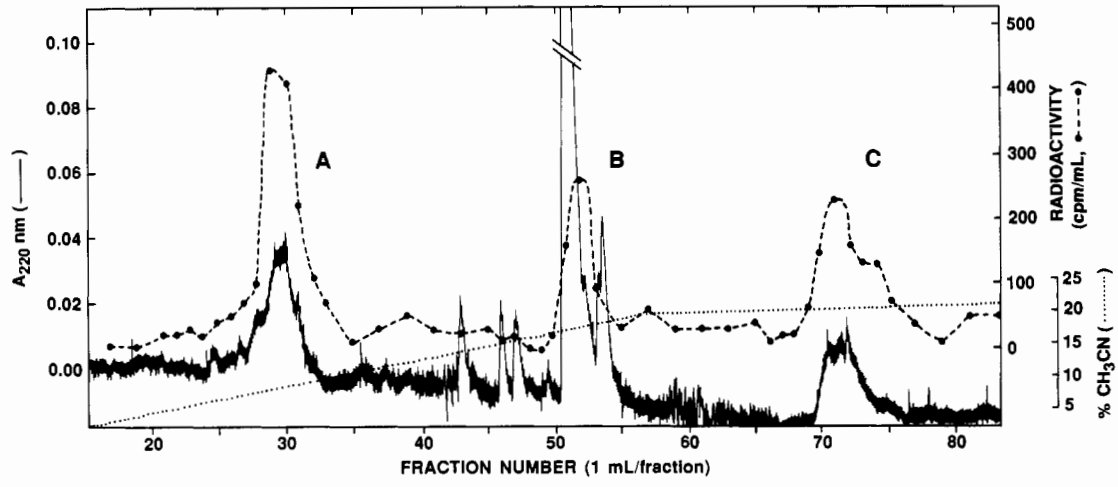

FIGURE 2: Peptides derived from modification of pyruvate kinase with no ligands present: fractionation by reverse-phase HPLC. The material that was eluted with water from the PBA-30 column was lyophilized and dissolved in 0.1% trifluoroacetic acid, filtered, and applied to the HPLC.

the column in pH 8.0 buffer, while the unmodified peptides are not retained. The diol–borate complex is dissociated by decreasing the pH or salt concentration (Annamalai et al., 1979). The majority of the peptides were eluted in the equilibrating buffer, 50 mM potassium phosphate, pH 8.0, as shown in Figure 1. The presence of some radioactivity in this peak was probably due to exchange of ^3H into the unmodified peptides. After the column was washed in the same buffer, the bound nucleotidyl peptides were eluted with deionized H_2O in a single sharp peak with a small A_{220nm} peak containing 83% of the recovered radioactivity. In the representative run illustrated, this peak (milliliters 134–140) was pooled and lyophilized for further purification. A final wash with 0.1 M ammonium acetate, pH 5.0 (not shown), eluted no additional radioactivity.

These modified peptides were dissolved in 0.1% trifluoroacetic acid and separated by HPLC in a water (0.1% in TFA)/acetonitrile (0.07% in TFA) gradient. Figure 2 illustrates a representative run showing the pattern of 220-nm absorbance and the distribution of radioactivity in the peptides derived from modified enzyme which contained 2 mol of reagent/mol of subunit. The three radioactive nucleotidyl peptide peaks are designated peaks A–C. Peak A coincides with an A_{220nm} peak centered at fraction 30 and represents 45% of the radioactivity of the peptide-associated tritium, while peak B contains 19% of the radioactivity. Peak C represents 31% of the radioactivity and coincides with an A_{220nm} peak centered at fraction 72. The radioactivity of peaks B and C together totals 50% of the radioactivity of peptide-associated tritium.

Characterization of Modified Peptides. Peak A contained a single peptide as indicated by the unique amino acid sequence (sample I) using the gas-phase sequencer (Table I). One nanomole of this peptide, prepared from pyruvate kinase

Table I: Amino Acid Sequence of Peptides A–C

	peptide A			peptide B[a]		peptide C[a]	
cycle	amino acid	sample 1[a] (pmol)	sample 2[b] (pmol)	amino acid	amount (pmol)	amino acid	amount (pmol)
1	Asn	990[c]	967	Asn	121[d]	X	
2	Ile	1100	1010	Thr	102	Asp	218[e]
3	X			Gly	73	Glu	209
4	Lys	578	552	Ile	125	Asn	185
5				Ile	127	Ile	207
6				X		Leu	234
7				Thr	55	Trp	111
8				Ile	82	Leu	213
9				Gly	54	Asp	89
10				Pro	57	Tyr	127
11				Ala	64	Lys	103
12				Ser	52		
13				Arg	*f*		

[a] Sample derived from digest of pyruvate kinase modified in the absence of ligands (as in Figure 2). [b] Sample derived from digest of pyruvate kinase modified in the presence of PEP, KCl, and $MnSO_4$ (as in Figure 3). [c] This peptide contained 1780 pmol of tritium on the basis of the specific radioactivity/hydrogen in the original [^{3}H]$NaBH_4$. [d] This peptide contained 151 pmol of tritium. [e] This peptide contained 690 pmol of tritium. [f] Although Arg was expected as the C-terminal residue of this tryptic peptide, the amount of sample remaining was too low to allow its detection.

modified in the absence of protectants, was applied to the sequencer, yielding Asn-Ile-X-Lys. This corresponds to residues 162–165 in the known amino acid sequence of cat muscle pyruvate kinase (Muirhead et al., 1986). The X at cycle 3 indicates that no phenylthiohydantoin derivative was detected in this cycle. Since the amino acid sequence of the cat muscle pyruvate kinase contains a cysteine at this position, the peptide is most likely modified at the cysteine. The amount of the peptide applied to the sequencer was 1100 pmol, and the amount of tritium it contained was 1780 pmol, giving a stoichiometry of 1.6 mol of ^{3}H/mol of amino acid, which is close to the amount expected for reduction of the two carbonyl groups of the dioxobutyl moiety considering a small kinetic isotope effect for the hydride transfer reaction (DeCamp & Colman, 1989). In the case of this peptide and all other BDB-nucleotide-modified peptides derived from pyruvate kinase, the radioactivity did not elute from the sequencer with any PTH amino acid but remained bound to the filter.

Peak C also contained a single peptide. The analysis of the modified peptide, shown in Table I, yielded the sequence X-Asp-Glu-Asn-Ile-Leu-Trp-Leu-Asp-Tyr-Lys, which is the same as residues 151–161 of the cat muscle enzyme. No phenylthiohydantoin derivative was detected in cycle 1, and because the cat muscle pyruvate kinase contains a cysteine at this position, peptide C is also in all probability modified at the cysteine. Since the first cycle showed no amino acid and the second and third contained amino acids that often give low yields on gas-phase sequencing, the amount of peptide loaded was estimated from the amount of leucine present in cycle 5, or 234 pmol. The peptide applied to the sequencer contained 690 pmol of tritium, giving a stoichiometry of 2.9 mol of tritium/mol of peptide. This figure probably corresponds to an actual stoichiometry of 2 mol of tritium/mol of peptide; the occurrence of the modified amino acid at the N-terminal position of peptide C may interfere with the Edman reaction, causing an abnormally low yield of PTH amino acid. Peaks A and C are both broad, yet each contains a single peptide sequence. This phenomenon has been observed previously (Hollemans et al., 1983; Jacobson & Colman, 1984; Batra & Colman, 1986; Ehrlich & Colman, 1987; Buechler & Taylor, 1988; DeCamp & Colman, 1989; Bansal et al., 1989), and the slight differences in retention times may be due to the loss of phosphate or to differences in the stereochemistry of reduction. Peptide B gave the sequence Asn-Thr-Gly-Ile-Ile-X-Thr-Ile-Gly-Pro-Ala-Ser-Arg and comigrated with another peptide under the conditions used for separation by HPLC. The comigrating peptide began with the sequence Met-Asn-Phe-Ser-His-Gly-Thr-His-Gly-Tyr-His-Ala-Glu-Thr-Ile and corresponded with the sequence of the cat muscle pyruvate kinase, Leu-Asn^{74}–Ile^{87}. Further purification of these peptides on HPLC was difficult due to their low solubility in several other solvents. It is probable that this second peptide was not labeled with tritium and therefore was not a nucleotidyl peptide because partial purification (in ammonium acetate, pH 5.8, with an acetonitrile gradient) resulted in a 5-fold decrease in the amount of comigrating peptide present but did not decrease the apparent specific radioactivity of peptide B. Peptide B yielded no PTH derivative in cycle 6; this position corresponds to a cysteine in the sequence of the cat muscle enzyme, Asn^{43}–Arg^{55}. Hence, the peptide is most likely modified at Cys^{48}. An average of 1.5 mol/mol was obtained for the ratio of tritium to peptide for two runs of 20 and 120 pmol of peptide.

Isolation of Peptide from Enzyme Incubated with 8-BDB-TA-5′-TP in the Presence of Protecting Ligands. Enzyme incubated with 175 μM 8-BDB-TA-5′-TP under protecting conditions for the same time period (80 min in the presence of 5 mM PEP, 100 mM KCl, and 2 mM $MnSO_4$) lost only 20% of its activity and incorporated only 1 mol of affinity label/mol of subunit. Under these conditions, peak A was the only radioactive peak present (Figure 3), and the enzyme retained 80% of its activity. A stoichiometry of 1 per subunit suggests complete reaction at the target residue in the radiolabeled peptide represented by peak A. That this is indeed peptide A is demonstrated by the identical amino acid sequences (Table I, samples 1 and 2) obtained from enzyme samples modified with 8-BDB-TA-5′-DP either in the absence or in the presence of protectants. In the absence of protectants, peaks B and C appear in addition, concomitant with the almost complete loss of activity and an increase by 1 mol/mol of subunit in the amount of reagent incorporated. Since the appearance of peaks B and C is associated with loss of activity of the pyruvate kinase, modification of one or both of these residues must be responsible for inactivation. Since their combined stoichiometries total 1, reaction at these two sites may be mutually exclusive, reaction at one site preventing reaction at the other site, while modification of either site may be sufficient to cause inactivation.

Discussion

8-[(4-Bromo-2,3-dioxobutyl)thio]adenosine 5′-triphosphate functions as a classical affinity label of the substrate binding site of pyruvate kinase. Although conceived as a nucleotide analogue, results of protection experiments showed PEP, KCl, and Mn^{2+} to be more effective at preventing inactivation of pyruvate kinase than is ADP or ATP. In light of the protection studies, 8-BDB-TA-5′-TP is considered to be a phosphoenolpyruvate analogue in the sense that the tautomer of the dioxobutyl group with one of the two oxo groups enolized has an α,β unsaturated keto group which is similar to the α,β unsaturated carboxyl of phosphoenolpyruvate and a hydroxyl group which is structurally equivalent to the phosphoryl group of phosphoenolpyruvate (DeCamp et al., 1988). Proton NMR spectra indicate that 1,4-dibromobutanedione, as well as the BDB nucleotides, exists at least partially in enolate and hydrated forms in polar solvents (Colman et al., 1984; Kapetanovic et al., 1985; Bailey & Colman, 1987). In support of the

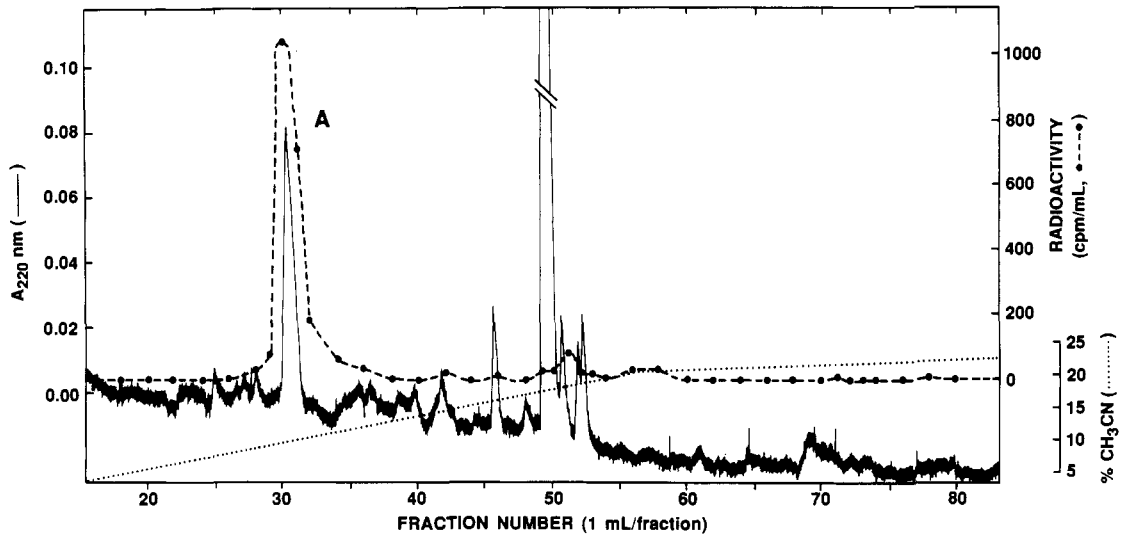

FIGURE 3: Peptides resulting from modification of pyruvate kinase in the presence of PEP, KCl, and $MnSO_4$: fractionation by reverse-phase HPLC. The material that eluted in water from the PBA-30 column was lyophilized and dissolved in 0.1% trifluoroacetic acid, filtered, and applied to the HPLC.

interpretation of 8-BDB-TA-5′-TP as a PEP analogue is the fact that 175 μM 8-[(3-bromo-2-oxopropyl)thio]-ATP (8-BOP-TA-5′-TP), which exists only in the keto form even in polar solvents (Bailey & Colman, 1987) and hence is "unlike" phosphoenolpyruvate, inactivates pyruvate kinase very slowly with a rate constant of 0.015 min^{-1} as compared with 0.23 min^{-1} for 8-BDB-TA-5′-TP under the same conditions (DeCamp, 1988). Similarly, the PEP analogue 2-BDB-TεA-5′-DP inactivates pyruvate kinase rapidly, while the same concentration of 2-BOP-TεA-5′-DP has little effect on activity (DeCamp & Colman, 1989). In contrast, 2-[(4-bromo-2,3-dioxobutyl)thio]adenosine 2′,5′-bisphosphate and 2-[(3-bromo-2-oxopropyl)thio]adenosine 2′,5′-bisphosphate, which function as affinity labels of the coenzyme site of $NADP^+$-dependent isocitrate dehydrogenase, inactivate that enzyme at comparable rates (Bailey & Colman, 1987).

The rate constant for inactivation of pyruvate kinase exhibits a nonlinear dependence on 8-BDB-TA-5′-TP concentration, consistent with the reversible formation of an enzyme–reagent complex prior to covalent reaction at the active site. Specific protection against inactivation is provided by substrates in the presence of manganese, consistent with the requirement for divalent metal. Activity loss to the extent of 97% correlates with a stoichiometric amount of reagent incorporation. Previous work in this laboratory (DeCamp & Colman, 1988) indicated that 2 mol of 8-BDB-TA-5′-TP were incorporated into pyruvate kinase but that only one residue was essential for activity. The nonessential residue was postulated to be a cysteine on the basis of quantification of the free cysteines with 5,5′-dithiobis(2-nitrobenzoate). We have now confirmed this suggestion, finding that one peptide (represented by peak A) is labeled under protecting conditions and have ascertained the modified residue to be Cys^{164}. 8-BDB-TA-5′-TP is identical with 2-BDB-TεA-5′-DP in its reaction with pyruvate kinase in the presence of protectants: they both react with the same nonessential residue, Cys^{164}, located in the tryptic tetrapeptide Asn^{162}-Ile-Cys-Lys^{165}. It is interesting that this nonessential residue is not the reactive cysteine of pyruvate kinase which is labeled most readily by iodoacetic acid (Harkins et al., 1983). Although Cys^{164} is not required for activity and is internal (Muirhead, 1987), it appears to be favorably positioned for reaction with nucleotide analogues.

Peaks B and C contain the radioactive peptides labeled by 8-BDB-TA-5′-TP which are absent under protecting conditions and are responsible for loss of activity. These peaks contribute, respectively, 19% and 31% of the peptide-bound radioactivity, totaling together half of the peptide-bound radioactivity or about 1 mol/mol of enzyme subunit. Reaction at either of these sites appears to cause loss of activity, and the two reactions may be mutually exclusive: for a given subunit, reaction with either residue may preclude further reaction within that subunit. Gas-phase sequencing revealed a 13-residue peptide for peptide B containing Cys^{48} and an 11-residue peptide for peptide C starting with Cys^{151}. No phenylthiohydantoin amino acids were detected at cycles 6 and 1 for peptides B and C, respectively. Since cysteines are present in these positions in the cat muscle enzyme, it is highly likely that cysteines at these positions are the targets of the affinity label. The 8-BDB-TA-5′-TP-modified cysteine or its PTH derivative appears to decompose under the acid conditions encountered in gas-phase sequencing. A similar result has been observed when Cys^{164} reacts with 2-BDB-TεA-5′-DP (DeCamp & Colman, 1989).

In the reaction of the affinity label 2-BDB-TεA-5′-DP with pyruvate kinase, modification of Tyr^{147} was shown to inactivate the enzyme; incorporation of 1.7 mol/mol of enzyme subunit accompanied 90% inactivation at 200 μM 2-BDB-TεA-5′-DP in 80 min. Protection against inactivation and against modification of Tyr^{147} was provided by PEP, KCl, and $MnSO_4$, resulting in only 1.0 mol of reagent incorporated (at Cys^{164}) when the enzyme was 14% inactivated. These results indicate that although 2-BDB-TεA-5′-DP reacted with two groups on the enzyme, reaction with only Tyr^{147}, in the region of the PEP binding site, caused substantial loss of activity.

We report here that $cysteine^{151}$, rather than Tyr^{147}, is the major residue responsible for inactivation in the reaction of 8-BDB-TA-5′-TP with pyruvate kinase. The distance between the α-carbons of Cys^{151} and Tyr^{147} in the crystal structure of pyruvate kinase is 8.9 Å. Since the target residues of the two affinity labels are approximately 9 Å apart, the question arises as to whether, if the nucleotide moieties were superimposed, the reactive CH_2Br groups would be situated 9 Å apart. Nucleotides featuring bulky substituents at the 8-position of the purine ring are known to exist predominantly in the syn conformation when in solution, whereas for nucleotides with 2-substituents, the anti conformation predominates (Czarnecki, 1984; Saenger, 1984; King et al., 1989). Given that 8-BDB-TA-5′-TP is in the syn conformation while 2-BDB-TεA-5′-DP

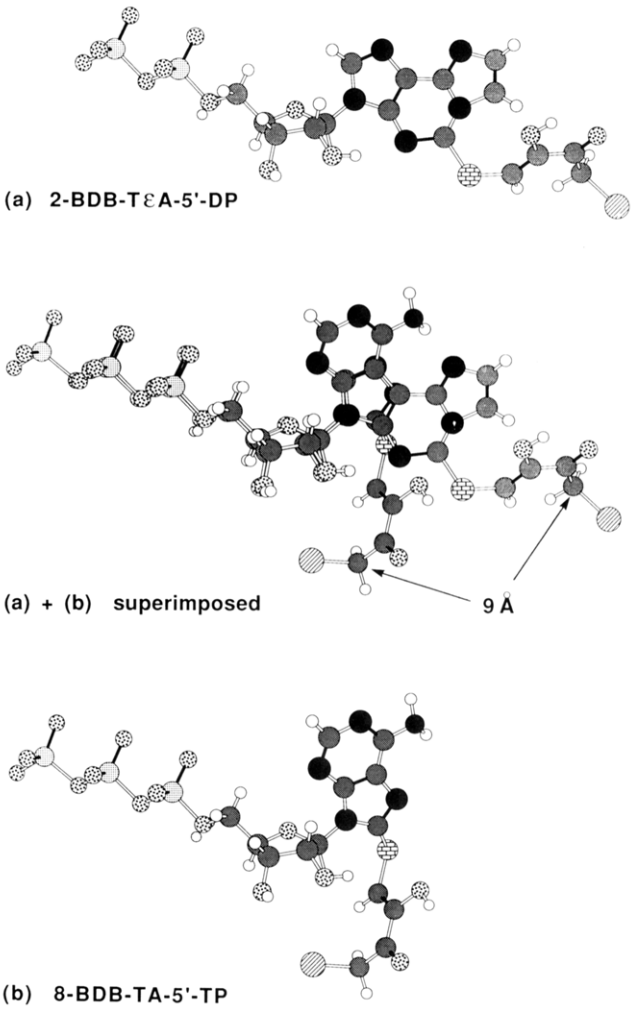

FIGURE 4: Comparison of structures of 2-BDB-TεA-5′-DP (a) and 8-BDB-TA-5′-TP (b). The nucleotide analogue 2-BDB-TεA-5′-DP has been positioned in an anti conformation with the preferable torsion angle of 210°, defined by O_4'–C_1'–N_1–C_2 (Saenger, 1984), and 8-BDB-TA-5′-TP has been placed in a syn conformation with the preferable angle of 30°. In the middle, the two structures are superimposed so that the ribose and phosphates coincide. The distance labeled is that between the carbon atoms of the reactive CH_2Br on the two molecules.

is in the anti conformation, it is easy to position the two compounds so that the distance between the reactive CH_2Br groups of 8-BDB-TA-5′-TP and 2-BDB-TεA-5′-DP is 9 Å. Figure 4 shows the conformation of the individual affinity labels and their appearance when superimposed. Although a range of distances between the two CH_2Br groups is possible, it is energetically permissible to arrange the compounds on the enzyme such that the distance between the reactive groups coincides with the 9-Å distance between Cys^{151} and Tyr^{147}. If the purine, ribose, and phosphates bind similarly to the enzyme, the experimental results from affinity labeling are consistent with the atomic positions assumed by the enzyme in the crystalline form. The precise positioning of the analogue on the enzyme is probably determined by multiple interactions of the nucleotide and enolized bromodioxobutyl groups with the protein.

This study is the first to report loss of activity associated with the chemical modification of Cys^{151} or Cys^{48}, but Cys^{151} had previously been located at the entrance to the active site (Muirhead, 1987). Cys^{48} is an internal cysteine (Muirhead et al., 1986). It is difficult to understand why Cys^{48} is a target of 8-BDB-TA-5′-TP since it is located in the A domain about 36 Å from the α-carbon of Cys^{151}, distant from the region designated the active site [the cleft between the A and B domains (Muirhead et al., 1986)] and too far from the PEP site to be directly protected by this substrate. It is possible that a conformational change induced by the binding of PEP to pyruvate kinase may account for protection of Cys^{48} by PEP. Similarly, reaction of Cys^{151} with 8-BDB-TA-5′-TP may induce the same conformational change as does PEP, thereby protecting Cys^{48} within a given subunit and rendering the reactions at these two cysteines mutually exclusive. This explanation could account for the stoichiometry of one obtained for the sum of the reactions at these two residues, coincident with loss of activity. Additionally, Cys^{48} is somewhat closer to the region designated the second nucleotide binding site (Mildvan & Cohn, 1986; Stuart et al., 1979; Rosevear et al., 1987), and it is possible that this binding site recognizes the nucleotide portion of 8-BDB-TA-5′-TP.

REFERENCES

Annamalai, A. E., Pal, P. K., & Colman, R. F. (1979) *Anal. Biochem. 99*, 85–91.

Bailey, J. M., & Colman, R. F. (1987) *Biochemistry 26*, 6858–6869.

Bansal, A., Dayton, M. A., Zalkin, H., & Colman, R. F. (1989) *J. Biol. Chem. 264*, 9827–9835.

Batra, S. P., & Colman, R. F. (1986) *Biochemistry 25*, 3508–3515.

Bucher, T., & Pfleiderer, G. (1955) *Methods Enzymol. 1*, 435–440.

Buechler, J. A., & Taylor, S. S. (1988) *Biochemistry 27*, 7356–7361.

Colman, R. F., Huang, Y.-C., King, M. M., & Erb, M. (1984) *Biochemistry 23*, 3281–3286.

Cottam, G., Hollenberg, P., & Coon, M. J. (1969) *J. Biol. Chem. 244*, 1481–1486.

Czarnecki, J., Geahlen, R., & Haley, B. E. (1979) *Methods Enzymol. 56*, 642–653.

DeCamp, D. L. (1988) Ph.D. Dissertation, University of Delaware, Newark, DE.

DeCamp, D. L., & Colman, R. F. (1989) *J. Biol. Chem. 264*, 8430–8441.

DeCamp, D. L., Lim, S., & Colman, R. F. (1988) *Biochemistry 27*, 7651–7658.

Dunaway-Mariano, D., Benovic, J. L., Cleland, W. W., Gupta, R. K., & Mildvan, A. S. (1979) *Biochemistry 18*, 4347–4354.

Ehrlich, R. S., & Colman, R. F. (1987) *J. Biol. Chem. 262*, 12614–12619.

Gupta, R. K., Fung, C. H., & Mildvan, A. S. (1976) *J. Biol. Chem. 251*, 2421–2430.

Harkins, R. N., Nocton, J. C., Russell, M. P., Fothergill, L. A., & Muirhead, H. (1983) *Eur. J. Biochem. 136*, 341–346.

Hollemans, M., Runswick, M. J., Fearnley, I. M., & Walker, J. E. (1983) *J. Biol. Chem. 258*, 9307–9313.

Jacobson, M. A., & Colman, R. F. (1984) *Biochemistry 23*, 6377–6382.

Kapetanovic, E., Bailey, J. M., & Colman, R. F. (1985) *Biochemistry 24*, 7586–7593.

King, S. M., Haley, B. E., & Witman, G. B. (1989) *J. Biol. Chem. 264*, 10210–10218.

Mildvan, A. S., & Cohn, M. (1966) *J. Biol. Chem. 241*, 1178–1193.

Mildvan, A. S., Sloan, D. L., Fung, C. H., Gupta, R. K., & Melamud, E. (1976) *J. Biol. Chem. 251*, 2431–2434.

Muirhead, H. (1987) in *Biological Macromolecules and Assemblies* (Jurnak, F. A., & McPherson, A., Eds.) Vol. 3, pp 143–186, Wiley, New York.

Muirhead, H., Clayden, D. A., Barford, D., Lorimer, C. G., Fothergill-Gilmore, L. A., Schlitz, E., & Schmitt, E. (1986) *EMBO J. 5*, 475–481.
Nageswara Rao, B. D., Kayne, F. J., & Cohn, M. (1979) *J. Biol. Chem. 254*, 2689–2696.
Nowak, T., & Mildvan, A. S. (1972) *Biochemistry 11*, 2819–2828.
Penefsky, H. S. (1979) *Methods Enzymol. 56*, 527–530.
Rosevear, P. R., Fox, T. L., & Mildvan, A. S. (1987) *Biochemistry 26*, 3487–3493.
Saenger, W. (1984) *Principles of Nucleic Acid Structure*, pp 22, 69–82, Springer-Verlag, New York.
Stuart, D. I., Levine, M., Muirhead, H., & Stammers, D. K. (1979) *J. Mol. Biol. 134*, 109–142.
Vollmer, S. H., & Colman, R. F. (1989) *The Protein Society, Third Symposium*, August, 1989, Seattle, WA.

Evidence for an Inhibitory Effect Exerted by Yeast NMN Adenylyltransferase on Poly(ADP-ribose) Polymerase Activity

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Received June 12, 1989; Revised Manuscript Received October 17, 1989

ABSTRACT: We have previously reported for the first time the purification to homogeneity of the enzyme NMN adenylyltransferase (EC 2.7.7.1) from yeast and its major molecular and catalytic properties. The homogeneous enzyme was found to be a glycoprotein containing 2% carbohydrate and 1 mol of adenine residue and 2 mol of phosphate covalently bound per mole of protein. Such a stoichiometry, apparently consistent with that of ADP-ribose, prompted us to further investigate the possibility that NMN adenylyltransferase could be subjected to poly(ADP-ribosylation) in vitro in a reconstituted system. Poly(ADP-ribose) polymerase was purified to homogeneity from bull testis by means of a rapid procedure involving two batchwise steps on DNA–agarose and Reactive Blue 2 cross-linked agarose and a column affinity chromatography step on 3-aminobenzamide–Sepharose; the optimal conditions for the poly(ADP-ribosylation) of exogenous substrates were determined. When pure NMN adenylyltransferase was incubated in the presence of the homogeneous poly(ADP-ribose) polymerase, a marked inhibition of the polymerase was observed, both in the presence and in the absence of histones, while the activity of NMN adenylyltransferase was not affected. The inhibition could not be prevented by increasing the concentrations of either DNA or NAD. Mg^{2+} did not affect the activity or the inhibition. The significance of such a phenomenon is at present unknown, but it may be of biological relevance in view of the close topological and metabolic relationship between the two enzymes.

In addition to its universal function as a cofactor in oxidation–reduction reactions, NAD is known to be utilized as a substrate in ADP-ribosylation reactions. Quite uniquely the NAD synthesis from NMN and ATP is localized in the nucleus, catalyzed by the enzyme NMN adenylyltransferase (EC 2.7.7.1) strictly bound to chromatin (Siebert, 1963). The enzyme poly(ADP-ribose) polymerase (EC 2.4.2.30) catalyzes the polymerization of ADP-ribose moieties from NAD on target nuclear proteins and histones, as well as on the enzyme itself (automodification), resulting in NAD breakdown. Such a posttranslational modification has been related to many different nuclear events, such as differentiation, cell division, and DNA repair (Shall, 1985). Because of its ability to freely cross the nuclear envelope (Siebert, 1972), any depletion of NAD caused in the nucleus by the occurrence of events involving enhanced ADP-ribosylation is readily transferred as a signal to the cytoplasm, where NAD-related metabolic reactions can be accordingly regulated, and vice-versa (Loetscher et al., 1982). Despite the increasing interest in the physiological role of the poly(ADP-ribosylation) level, as well as in the identification of specific enzyme protein acceptors (Gaal & Pearson, 1985; Althaus & Richter, 1988), less interest has been focused on the other nuclear enzyme of NAD metabolism, NMNAT,[1] although it represents a key route for the synthesis of NAD within the cell.

On the basis of indirect evidence, a direct relationship between this enzyme and ADPRP has been suggested (Uhr & Smulson, 1982). To investigate its regulatory properties, we have purified for the first time to homogeneity the enzyme NMNAT from yeast (Natalini et al., 1986) and found evidence for its possible ADP-ribosylation; consistent with observations by others (Miwa et al., 1983; Mura, 1987), we also demonstrated in purified yeast nuclei the presence of an ADP-ribosylating activity, together with degrading activities both inactivating the ADPRP enzyme and hydrolyzing the

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[1] Abbreviations: NMNAT, NMN adenylyltransferase; ADPRP, poly(ADP-ribose) polymerase; ADPR, ADP-ribose; FPLC, fast protein liquid chromatography; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; TCA, trichloroacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; 3-ABA, 3-aminobenzamide; 3-MBA, 3-methoxybenzamide; BSA, bovine serum albumin.

0006-2960/90/0429-2501$02.50/0