Identification of Tyrosine and Lysine Peptides Labeled by 5′-p-Fluorosulfonylbenzoyl Adenosine in the Active Site of Pyruvate Kinase

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The nucleotide affinity label 5′-p-fluorosulfonylbenzoyl adenosine reacts at the active site of rabbit muscle pyruvate kinase, with irreversible inactivation occurring concomitant with incorporation of about 1 mol of reagent/mol of enzyme subunit (Annamalai, A. E., and Colman, R. F. (1981) J. Biol. Chem. 256, 10276–10283). Purified peptides have now been isolated from 70% inactivated enzyme containing 0.7 mol of reagent/mol of enzyme subunit. Rabbit muscle enzyme labeled with radioactive 5′-p-fluorosulfonylbenzoyl adenosine was digested with thermolysin. Nucleosidil peptides were purified by chromatography on phenylboronate-agarose and reverse-phase high performance liquid chromatography. After amino acid and N-terminal analysis, the peptides were identified by comparison with the primary sequences of chicken and cat muscle enzyme. About 75% of the reagent incorporated was distributed equally among three O-(4-carboxybenzenesulfonyl)tyrosine-containing peptides: Leu-Asp-CBS-Tyr-Lys-Asn, Val-CBS-Tyr, and Leu-Asp-Asn-Ala-CBS-Tyr. These tyrosines are located in a 28-residue segment of the 530-amino acid sequence. The remainder of the incorporation was found in two N-(4-carboxybenzenesulfonyl)lysine-containing peptides: Leu-CBS-Lys and Ala-CBS-Lys-Gly-Asp-Tyr-Pro. Modification in the presence of MnATP or MnADP resulted in a marked decrease in labeling of these peptides in proportion to the decreased inactivation. It is suggested that these modified residues are located in the region of the catalytically functional nucleotide binding site of pyruvate kinase.

EXPERIMENTAL PROCEDURES

Materials—Rabbit muscle pyruvate kinase was purchased from Boehringer Mannheim as an ammonium sulfate suspension. The enzyme was dialyzed overnight at 4 °C against 50 mM potassium phosphate buffer, pH 7.4, containing 1 mM dithiothreitol, centrifuged for 10 min at 33,000 rpm, and stored at −85 °C. The dithiothreitol was removed from the enzyme prior to reaction with 5′-FSBA by column centrifugation using Sephadex G-50-80 (14). The enzyme concentration was determined by using $E_{280nm} = 0.54$ (15) and an $A_m$ of 237,000/tetramer (16). The specific activity was 250–300 units/mg, where a unit is defined as the oxidation of 1 pmol of NADH/min in the coupled assay (see below).

Tritiated 5′-p-fluorosulfonylbenzoyl adenosine (5′-[3H]FSBA) was prepared from [2-3H]adenosine (New England Nuclear) by the

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method of Wyatt and Colman (11). The specific radioactivity was 1.04 \times 10^{22} \text{ cpm/mol}. The concentration was measured spectrophotometrically using \(c_{\text{protein}} = 1.35 \times 10^{-4} \text{ M}^{-1} \text{ cm}^{-1}\) (11).

Phosphoenolpyruvate (tricyclohexylammonium salt), ATP, ADP, NADH, thymol, and dansyl chloride were supplied by Sigma. Lactate dehydrogenase (hog muscle) was purchased from Boehringer Mannheim and used without further purification. Monate Gel PBA-10 was from Amicon and HPLC-grade acetonitrile was from Burdick and Jackson Laboratories, Inc. Schleicher & Schuell micropolyamide sheets and trifluoroacetic acid were purchased from Pierce Chemical Co.

 Assay for Enzyme Activity—Pyruvate kinase activity was measured spectrophotometrically at 340 nm by a coupled assay with lactate dehydrogenase. The enzymatic activity was monitored at 30 °C in 50 mM Tris-HCl buffer, pH 7.5, containing 100 mM KCl, 10 mM MgSO\(_4\), 0.5 mM phosphoenolpyruvate, 3 mM ADP, 0.2% NaN\(_3\), and lactate dehydrogenase at a concentration of 0.1 mg/ml.

The volume of assay solution in the cuvette was 1 ml.

 Modification of Rabbit Muscle Pyruvate Kinase with 5'-FSBA—Pyruvate kinase (5 mg/ml) was incubated with 2 mM 5'-[\text{3H}]FSBA at 30 °C in 0.1 M MOPS buffer, pH 7.4, containing 0.2 mM KCl and 15% dimethyl formamide. In one reaction, 20–50 mg of protein were modified. Residual activity after reactivation by dithiothreitol (DTT) was evaluated at intervals by withdrawing a 50-μl aliquot from the reaction mixture, adding 0.1 M DTT to give a final concentration of 0.02 M DTT, and incubating for 1 h at 30 °C. Thereafter, 20 μl was withdrawn and diluted 1600 times with 0.01 M potassium phosphate buffer, and the activity of 20 μl was measured in the assay solution.

For each reaction mixture, a corresponding control was run in which 5'-FSBA was absent. All controls retained their original specific activity throughout the experiment. Residual activity was calculated from the ratio of the measured enzyme activity for the reaction mixture containing 5'-FSBA (E) to the measured enzyme activity for the corresponding control reaction mixture (E).

 Reactivation and Isolation of Modified Pyruvate Kinase—After 6 h, 0.1 M DTT was added to the remaining enzyme solution to give a final concentration of 0.02 M DTT. After a 1-h incubation at 30 °C, solid urea was added to give a concentration of 6 M. Excess reagent was removed from the denatured enzyme by a modification of the Penefsky method (14). Five-ml plastic syringes (Plastipak 5603, Beckman Instruments, Fullerton, CA) were filled with Sephadex G-50-80 equilibrated with 0.05 M potassium phosphate, pH 8, containing 6 M urea and 1 mM DTT, and spun in a tabletop centrifuge. The reaction mixture was divided into 1-ml aliquots, and each was applied to one "mini-column." The effluents of all the mini-columns were pooled and dialyzed overnight against 0.05 M potassium phosphate, pH 8, containing 6 M urea and 1 mM DTT.

Measurement of Incorporation of SBA into Pyruvate Kinase—The protein concentration of the dialyzed aliquot was measured using the Bio-Rad assay, which is based on the method of Bradford (17). The incorporation of 5'-[\text{3H}]FSBA was determined by counting an aliquot of modified enzyme in 10 ml of ACS (Amersham) using a Packard Tri-Carb liquid scintillation counter, Model 3330.

Proteolytic Digestion of Modified Pyruvate Kinase—After dialysis, the modified enzyme was carboxymethylated. DTT was added to give a 10-fold excess over protein sulphydryl groups. After 1 h, a 2-fold excess was added of iodoacetic acid over the concentration of sulphydryl groups in the protein plus the DTT. Ten minutes later, the reaction was quenched by the addition of excess β-mercaptoethanol. The carboxymethylated enzyme was disulfated against 0.05 M ammonium bicarbonate, pH 8, to remove excess reagents and urea before digestion. Thermolysin was added in a 1:20 (w/w) ratio to pyruvate kinase. The digest was incubated for 4 h at 37 °C, lyophilized, and stored at −80 °C.

Purification of Modified Peptides on Boronate Column—The SBA peptides were initially purified on a phenylboronate-agarose (PBA-10) column (1 x 27 cm) equilibrated with the starting buffer, 0.05 M potassium phosphate, pH 8. The non-nucleosidyl peptides were eluted with the starting buffer. Subsequent elution with deionized water removed the -SBA peptides. Any residual material was removed by 0.1 M ammonium bicarbonate, pH 5.0. Column fractions (1 ml) were monitored for 220 nm absorbance and for radioactivity by counting aliquots as described above. Appropriate fractions were pooled and lyophilized.

Separation of Modified Peptides by HPLC—Samples were subjected to high performance liquid chromatography on a Varian 5000 LC equipped with a Varichrom absorbance monitor and a Micropak MCH-10 C\(_\text{18}\) column (0.4 x 30 cm). The solvent system was 0.1% trifluoroacetic acid (Solvent A) and acetonitrile containing 0.07% trifluoroacetic acid (Solvent B) with a linear gradient of 0–30% acetonitrile in 120 min. The flow rate was 1 ml/min, and fractions of 1 ml were collected. The effluent was monitored continuously for absorbance at 220 nm and for radioactivity by counting aliquots of the fractions as described above.

 Analysis of Isolated Peptides—Peptides isolated from HPLC were subjected to amino acid analysis and N-terminal analysis. Samples for amino acid analysis were hydrolyzed for 24 h at 110 °C in 6 N HCl and run on a Beckman Model 120C modified for use as a one-column amino acid analyzer as described by Likos and Colman (18). Standard solutions of amino acids were purchased from Pierce Chemical Co., and stock solutions of CBS-Lys and CBS-Tyr were provided by Saradambal et al. (19). N-terminal residues were identified by dansylation and thin-layer chromatography on polyamide sheets (20).

RESULTS

Inactivation of Pyruvate Kinase by 5'-p-Fluorosulfonylbenzoyl Adenosine—Rabbit muscle pyruvate kinase is inactivated upon incubation with 2 mM 5'-FSBA at 30 °C in 0.1 M MOPS buffer, pH 7.4. A plot of E/E\(_0\) for the DTT-insensitive reaction is shown in Fig. 1, line 1. After a 6-h incubation, the modified enzyme contained 0.67 mol of sulfonylbenzoyl adenosine/mol of subunit and was about 70% inactivated. It was previously reported by Wyatt and Colman (11) that millimolar concentrations of ADP or ATP and Mg\(^{2+}\) give significant protection against loss of activity. Further studies by Annamalai and Colman (12) supported the postulate that residues within the metal-nucleotide binding site are labeled by 5'-FSBA. In the current study, the presence of 10 mM ADP or ATP and 12 mM MnCl\(_2\) resulted in a marked decrease in the extent of inactivation at any given time (Fig. 1, line 2); e.g., after 6 h, the enzyme was only 25% inactivated in the presence of ligands as compared to about 70% in their absence. Labeled peptides were isolated and characterized from enzyme incubated with 5'-FSBA in the absence of protectants and in the presence of Mn\(^{2+}\) and either ADP or ATP.

Initial Isolation of 5'-FSBA-modified Peptides—It was previously reported by Annamalai et al. (21) that dihydroxyboryl...

![Fig. 1. Inactivation of pyruvate kinase by 5'-FSBA in the absence and presence of ligands. Pyruvate kinase (5 mg/ml) was incubated with 2 mM 5'-FSBA at 30 °C in 0.1 M MOPS buffer, pH 7.4, containing 0.2 M KCl and 15% dimethyl formamide. Residual activity, E/E\(_0\), was measured as described under "Experimental Procedures." Line 1, no additions to reaction mixture (●). Line 2, either 10 mM ADP and 12 mM MnCl\(_2\) (○) or 10 mM ATP and 12 mM MnCl\(_2\) (●) added to the reaction mixture.](image-url)
substituted polyacrylamide or cellulose columns can be used to purify peptides modified with fluorosulfonylbenzoyl nucleotide analogs. The cis-diol moiety of the analog forms a reversible complex with the boronate ligands of the resin, while the majority of the unmodified peptides are eluted in the void volume. In the present study, a phenylboronate-to-purify peptides modified with fluorosulfonylbenzoyl nu-

te absorbing and radioactive peaks is

phosphate buffer and applied to a PBA-10 column. The column was

ADP and 12 mM MnCl2. The chromato-

pH 8, in the void volume along with about 25% of the

pyruvate kinase were eluted with 50 mM phosphate buffer,

agarose column was found to give the best recovery of the

while the majority of the unmodified peptides are eluted in

reversible complex with the boronate ligands of the resin,

was pooled and lyophilized for further purification. A final

modified enzyme; comparable amounts

of radioactivity have been applied to

HPLC. Fig. 3A shows the chromatogram of peptides

is hydrolyzed, with a half-life of 43 min, to fluores-

and the corresponding sulfonic acid by incubation at pH

at room temperature. This treatment resulted in the quanti-

tative removal of the radioactive adenine moiety from the

modified peptides, with the formation of carboxybenzenesul-

diol moiety of the analog forms a

peptide distribution. Only that

shown.

washed with deionized water to elute the bound peptides. A single,

sharp peak with a small absorbance at 220 nm and

about 54% of the recovered radioactivity resulted; this peak

was pooled and lyophilized for further purification. A final

was filtered. Aliquots containing 70-90 nmol

of the peptide distribution. Only that

shown.

peptide pool from modification with no

ligands present, as described under "Ex-

perimental Procedures." B, pool from modifi-

cation in the presence of 10 mM

ADP and 12 mM MnCl2. The chromatograms

shown in A and B result from the purifi-

cation of different amounts of modified enzyme; comparable amounts

of radioactivity have been applied to

HPLC in each case to allow comparison

of the peptide distribution. Only that

portion of the chromatogram containing

UV-absorbing and radioactive peaks is shown.

Since the ester bond of 5'-FSBA is not stable to prolonged

incubation in 0.1% trifluoroacetic acid, before being subjected to high performance liquid chromatography in that solvent, the modified peptides were treated with 0.3 N NH2OH for 4 h at room temperature. This treatment resulted in the quanti-

tative removal of the radioactive adenine moiety from the

modified peptides, with the formation of carboxybenzenesul-

fornyl-derivated peptides.

Fractionation of -SBA-labeled Peptides by HPLC—The base-hydrolyzed, modified peptides were lyophilized, redissolved in 0.1% trifluoroacetic acid, and subjected to reverse-phase HPLC. Fig. 3A shows the chromatogram of peptides isolated from modified enzyme which contained about 0.8 mol of -SBA/mol subunit. All 220 nm absorbing peaks were examined for radioactivity and for CBS-residues by amino acid analysis after hydrolysis in 6 N HCl. Peaks I through V were the only ones found to contain CBS-amino acids.

The recovery of radioactivity from the HPLC was 75-80%; of this, about 94% was associated with the adenine peak eluting at 16 min. The only other radioactive peak eluted at 54 min, exhibited marked absorbance at 220 nm, and contained about 6% of the recovered radioactivity. When this peak was subjected to amino acid analysis, only glycine and trace amounts of a few other amino acids were found. Since the 54-min peak appeared to be a decomposition product of the reagent, experiments were conducted to ascertain the elution times of 5'-FSBA and its decomposition products.

HPLC of Products of 5'-FSBA—The fluorosulfonyl moiety of 5'-FSBA is hydrolyzed, with a half-life of 43 min, to fluoride ion and the corresponding sulfonic acid by incubation at pH 8 (22). 5'-[2-H]FSBA was dissolved in 50 mM phosphate buffer, pH 8, containing 10% dimethyl formamide and was immediately subjected to the same HPLC program as was the peptide pool. A single peak of radioactivity and 220 nm absorbance emerged at 85 min, reflecting the elution of intact

![Graph](image-url)
5'-FSBA. After a 3-h incubation of 5'-FSBA at room temperature, HPLC showed a greatly reduced 85-min peak and a new, larger radioactive peak appearing at 39 min. The new peak is presumably the sulfonic acid derivative of 5'-FSBA. Alternatively, after 5'-FSBA was incubated for 4 h in 0.3 M NH$_4$OH, a single radioactive peak was observed at 54 min. The spectrum of this compound, presumably the sulfonamide formed by reaction of 5'-p-fluorosulfonylbenzoyl adenosine with ammonia, was identical with that of the unknown compound eluting in Fig. 3 at 54 min. This observation suggests that Tyr-147, Leu-158, and Val-173 in the cat enzyme are protected enzyme containing 0.25 mol of -SBA/mol of subunit previously. A chromatogram of peptides isolated from ADP-ATP kinase from chicken muscle (9). The only differences are that Tyr-147, Leu-158, and Val-173 in the chicken enzyme. 

Analysis of the Isolated Peptides—Peaks I–V were analyzed for amino acid content and for N-terminal by dansylation, with the results summarized in Table I. Each analysis represents the average of four experiments, and each residue is normalized to the amount of CBS-Lys or CBS-Tyr in the peptide.

From comparison of the data in Table I with the known amino acid sequence of cat muscle pyruvate kinase, the isolated peptides are identified as the following segments: I, NH$_2$-Leu-CBS-Lys-COOH (residues 228–229); II, NH$_2$-Ala-CBS-Lys-Gly-Asp-Tyr-Pro-COOH (residues 365–370); III, NH$_2$-Leu-Asp-CBS-Tyr-Lys-Asn-COOH (residues 158–162); IV, NH$_2$-Val-CBS-Tyr-COOH (residues 173–174); and V, NH$_2$-Leu-Asn-Ala-CBS-Tyr-COOH (residues 143–147). Comparative analysis can be located in the sequence of pyruvate kinase from chicken muscle (9). The only differences are that Tyr-147, Leu-158, and Val-173 in the cat enzyme are Phe, Val, and Ile, respectively, in the chicken enzyme.

Effect of Protecting Ligands on Peptide Distribution—To evaluate whether the protecting ligands influence the distribution of peptides labeled by 5'-FSBA, pyruvate kinase was incubated with 5'-FSBA in the presence of 12 mM MnCl$_2$ and 10 mM ADP or ATP, the protein was digested with thermolysin, and the modified peptides were isolated as described previously. A chromatogram of peptides isolated from ADP-protected enzyme containing 0.25 mol of -SBA/mol of subunit is shown in Fig. 3B. A comparison of Fig. 3B with 3A reveals that, although the relative amounts of some of the peptides are changed, all 5 are still present. The distribution of peptides isolated from modified enzyme with and without protection by metal-nucleotides is summarized in Table II. For each condition, the number of mol of -SBA incorporated into each peptide per mol of subunit is listed; these values were calculated by multiplying the total incorporation by the fractional representation of each peptide, which was determined by dividing the nmol of CBS-residues in each peptide by the total nmol of CBS-residues in all 5 peptides as measured by amino acid analysis. Labeling of all peptides is decreased by the presence of Mn$^{2+}$ and ADP or ATP.

**DISCUSSION**

We have isolated five unique peptides from the thermolysin digest of rabbit muscle pyruvate kinase modified by 5'-p-fluorosulfonylbenzoyl adenosine and identified them by comparison with the known cat muscle sequence (10). Three of the modified residues are tyrosines 147, 160, and 174, which are located in a 28-residue segment of the 530-amino acid sequence and together account for about 74% of the incorporated label. The remainder of the incorporation is due to the modification of lysines 229 and 366.

5'-FSBA reacts with rabbit muscle pyruvate kinase in a limited and specific manner: loss of enzymatic activity is directly proportional to the total incorporation of 1 mol of radioactive label/mol of peptide chain (11, 12). Modification of any single peptide is too small to account for the inactivation observed. Since loss of activity appears to depend on the total incorporation (12), the modification of any one of the 5 residues must result in an inactive enzyme, and reaction at 1 residue must exclude reaction at the other residues.

Although the distribution of reagent-labeled peptides changes somewhat in the presence of the metal and nucleotide, the most striking observation is that the modification of all the peptides is reduced in the presence of the substrates MnADP or MnATP. These results indicate that all the residues attacked by 5'-FSBA are located at or near the nucleotide binding site.

| Table I  
Analysis of the isolated peptides |
|-----------------------------------|
| Amino acid$^a$ | I | II | Residues/peptide$^a$ |
|----------------|---|---|-------------------|
| CBS-Lys$^b$    | 1.00 (1)$^d$ | 1.00 (1) |                       |
| CBS-Tyr        | 0.11 (0) | 0.18 (1) |                       |
| Asp            | 0.03 (0) | 0.15 (1) | 0.12 (0)             |
| Ser            | 0.06 (0) | 0.31 (0) | 0.19 (0)             |
| Glu            | 0.08 (0) | 0.76 (1) |                       |
| Pro            | 0.29 (0) | 1.07 (1) | 0.22 (0)             |
| Gly            | 0.08 (0) | 0.80 (1) | 0.28 (0)             |
| Ala            | 0.30 (0) | 0.41 (1) | 0.97 (1)             |
| Val            | 0.05 (0) | 0.64 (1) | 0.14 (0)             |
| Lys            | 0.22 (0) | 0.41 (1) | 0.97 (1)             |
| N-terminal     | Dansyl-Leu | Dansyl-Ala | Dansyl-Leu | Dansyl-Val | Dansyl-Leu |

$^a$ Each analysis represents the average of 4 experiments, with the amount of peptide ranging from 2.4–12.5 nmol in individual experiments.

$^b$ Met and Arg were not detected.

$^c$ Corrected by 15% for loss during hydrolysis (19) prior to normalization.

$^d$ Nearest integer.
Lys-366 has been previously modified by trinitrobenzenesulfonate, resulting in a complete loss of activity when 1 mol of trinitrobenzenesulfonate was incorporated per mol of subunit (23, 24). Hollenberg et al. (23) found that MgADP or MgATP gave strong protection against trinitrobenzenesulfonate-caused inactivation and that the $K_{in}$ values for phosphoenolpyruvate and ADP for the partially inactivated enzyme were similar to the constants obtained for the native enzyme. In addition, the modified enzyme was still able to bind phosphoenolpyruvate (25), to decarboxylate oxaloacetate (26), and to catalyze the phosphate-activated tritium exchange from pyruvate (25). The evidence suggested that Lys-366 is in or near the metal-ADP binding site. Peptide II, which accounts for 11% of the incorporation of FSBA, has the same sequence as a portion of the 34-amino acid trinitrophenyl-peptide isolated by Cardenas and co-workers (24) from bovine muscle.

Pyruvate kinase is known to have a broad specificity for nucleotide substrates; GDP and 1,4-N5-ethenoadenine give $V_{max}$ values which are comparable to that observed with ADP (27, 28). Muirhead and Clayden (10) have reported that their crystallographic studies suggest a nucleotide binding site which is generally large and nonspecific for adenine and that the main binding is through the phosphate groups.

The nuclear magnetic resonance studies of Mildvan and others (29, 30) have established that ATP binds to pyruvate kinase in an extended form with an anti-adenine ribose conformation. If 5′-FSBA binds in a similar manner, the reactive sulfonfonylfluoride moiety may attack residues which would normally be near the pyrophosphate of the nucleotide. Alternatively, the purine ring may be stacked with the benzoyl moiety, thereby placing the reactive group near residues in the purine-binding site. The NMR studies of Jacobson and Colman (31) indicate that 5′-FSBA and related nucleotides analogs undergo intramolecular stacking in solution. The two conformers may bind to pyruvate kinase in different ways, thereby accounting for the labeling of more than one amino acid. However, labeling by 5′-FSBA while in a stacked conformation must exclude labeling by 5′-FSBA in an extended conformation (and vice versa) since the total reaction is limited. According to Muirhead and Clayden, the adenine ring makes a number of hydrophobic interactions with Val-215, Val-220, and Phe-243. Lys-229 of peptide I is near these residues and possibly reacts with the label in its stacked conformation.

Inactivation of rabbit muscle pyruvate kinase is predominantly due to the approximately equal modification of three tyrosine residues. Likos et al. (32) also observed a 1.3 ratio of modified lysine to modified tyrosine residues when 5′-FSBA was used to modify the active site of yeast pyruvate kinase. The role of the tyrosines could involve hydrophobic interactions with adenine or hydrogen bonding with the phosphates. It is interesting to note that two of the three amino acid substitutions in the chicken as compared to the mammalian enzyme are conservative (i.e. Val for Leu, and Ile for Val), while Tyr-147 of peptide V is replaced by a phenylalanine in the chicken muscle pyruvate kinase (9), which may support the involvement of this residue in a hydrophobic interaction.

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