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RABBIT MUSCLE PYRUVATE KINASE

AMINO- AND CARBOXYL-TERMINAL STUDIES

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Summary

Amino-terminal analysis of rabbit muscle pyruvate kinase (ATP:pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) failed to detect the presence of any free amino-terminal residues. Acetyl group analysis demonstrated the presence of between 3.7 and 4.0 mol of acetyl groups per mol of enzyme. The acetylated amino-terminal residue was isolated from pronase digests of the enzyme and identified as *N*-acetylserine. Quantitative recovery experiments indicated that all acetyl residues are found at the amino termini. Carboxyl-terminal analyses using the tritium exchange method suggested the presence of a blocked carboxyl-terminal residue, supporting previous hydrazinolysis and carboxypeptidase studies.

Introduction

Rabbit muscle pyruvate kinase (ATP:pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) is a tetrameric protein [1,2]. Inactivation studies on the enzyme with 2,4,6-trinitrobenzene sulfonate indicated the presence of four essential lysyl ϵ-amino groups [3], and similar studies with 5,5′-dithiobis-(2-nitrobenzoate) indicated the presence of four essential sulfhydryl residues [4]. The location of these residues within the active site of the enzyme was demonstrated by NMR proton relaxation experiments [5]. In addition, the enzyme contains four binding sites for phospho*enol*pyruvate [6] as well as for univalent [6] and divalent cations [7,8].

Abbreviation: iPr_2P-F, diisopropylphosphorofluoridate.

In an attempt to further characterize the structure vs. function biochemistry of pyruvate kinase, we have studied the amino-terminal and carboxy-terminal residues of the enzyme. Experiments to determine the amino-terminal residues by a number of published procedures were unsuccessful. These findings, and the observation that the enzyme contains about 4 mol of acetate per mol of enzyme, suggested that the amino-terminal residue was acetylated [1]. This paper reports the isolation and identification of the acetylated amino acid as *N*-acetylserine.

Materials and Methods

Pyruvate kinase was prepared from frozen rabbit skeletal muscle by the method of Tietz and Ochoa [9]. After two or three recrystallizations from 0.020 M imidazole buffer, pH 7.0, containing $1 \cdot 10^{-3}$ M EDTA, the enzyme had a specific activity of about 330 units per mg protein and was homogeneous as judged by sedimentation and electrophoretic measurements [2].

The human fetal hemoglobin γ-chain was isolated as described by Stegink et al. [10] and the H_4 and M_4 lactate dehydrogenases were purchased from Sigma. Free acetate in the pronase preparation was removed by gel filtration (sephadex G-25), and the resulting preparation contained no protein-bound acetyl groups.

N-Acetyl-L-serine was purchased from Sigma. The *N*-[^{14}C]acetylserine was prepared by the method of Narita [11], yielding a mixture containing three parts *N*-acetylserine, one part *N,O*-diacetyl-L-serine and no detectable quantities of either free acetate or serine. The two acetylated derivatives were separated by high voltage electrophoresis. *O*-Acetylserine was prepared by the method of Sheehan et al. [12].

Tritiated water (5 Ci/g) was purchased from New England Nuclear, L-pyrrolidone carboxylate from Calbiochem, and carboxypeptidase A-iPr_2P-F and carboxypeptidase B-iPr_2P-F from Sigma.

Acetyl group analyses were carried out using the microenzymic method of Stegink [13]. Carboxyl-terminal analysis of intact pyruvate kinase, M_4 and H_4 lactate dehydrogenases, and human fetal hemoglobin γ-chain were carried out using a modification of the method of Holcomb et al. [14]. The labeled residues were released using either acid hydrolysis or digestion with carboxypeptidases [15]. The identity of released radioactive amino acids was determined using the simultaneous radioactivity and amino acid analysis technique described by Stegink [16].

The acetylated amino-terminal residue was isolated from pronase digests of pyruvate kinase using the method described originally by Narita [11,17] or a recent modification by Yoshida [18]. The enzyme was dialyzed against water to remove $(NH_4)_2SO_4$, and digested with pronase (6 mg, 20 h, 37°C, pH 7.8, 1 mM $CaCl_2$, 100 ml). The digest mixture was acidified to pH 3, passed over a Dowex 50-WX2 (H^+) column (Bio-Rad, 200—400 mesh, 2.4 × 20 cm) and washed with cold water until 200 ml of eluate were collected. The effluent was neutralized with base, lyophylized to dryness, and the residue dissolved in 0.025 M Tris buffer, pH 7.5 containing 0.1 M NaCl, followed by digestion (5 h, 37°C) with carboxypeptidase A-iPr_2P-F (10 units) and carboxypeptidase B-iPr_2P-F (50 units). The lyophilized digest was dissolved in water, acidified to pH 3 and passed through

a second Dowex-50 column to remove free amino acids.

High voltage electrophoresis was carried out on prewashed Whatman 3 MM paper in 10% acetic acid/0.5% pyridine for 3 h at 2.5 kV using a Savant flat-plate electrophoresis apparatus. Amino acids were detected with ninhydrin spray. Pyrrolidone carboxylate, pyrrolidone carboxylate peptides and acetylated amino acids and peptides were detected by the method of Rydon and Smith [19]. The positions of the radioactive derivatives were determined by a radio-isotope scanner.

Results

Previous studies demonstrated that rabbit muscle pyruvate kinase isolated in this laboratory failed to react with amino-terminal reagents, indicating the absence of a free amino-terminal residue [1]. These enzyme preparations contained 3.7—4 mol of *N*-acetyl groups per mol enzyme, whereas enzyme obtained from commercial sources contained only about 1 mol of acetyl group per mol enzyme, suggesting a lability of the acetyl group [1,13].

Initial studies using Narita's method [11,17] demonstrated that all acetyl groups present in the original enzyme were found in the Dowex-50 column effluent, indicating that no residues were present as ϵ-*N*-acetyllysine. High voltage electrophoresis of the Dowex-50 column effluent yielded a number of spots positive to the Rydon-Smith reagent [19], only one of which contained acetyl groups. Elution of this fraction from the electrophoretogram, followed by acid hydrolysis yielded approximately equimolar quantities of serine and acetate with smaller quantities of other amino acids, suggesting acetylserine to be the amino-terminal residue. However, recoevery of the acetyl groups originally present in pyruvate kinase as acetylserine was low.

N-Acetylserine is relatively labile under a number of conditions used in the chromatographic and high voltage purification of the acetylated derivative. To avoid such losses, the acetylated residues were isolated from pronase digests of pyruvate kinase using the modified method described by Yoshida [18]. Treatment of pyruvate kinase in this manner resulted in the isolation of a Dowex 50 fraction showing good recovery (73%) of all acetyl groups present in the original enzyme and which contained no ninhydrin positive material. Amino acid and acetyl group analysis after acid hydrolysis (Table I) showed stoichiometric quantities of acetate and serine, with smaller quantities of glutamate. The glutamate found after acid hydrolysis could arise either from a acetylated

TABLE I

COMPOSITION OF DOWEX 50 FRACTION ISOLATED CONTAINING *N*-ACETYL RESIDUES

Compound	μmol	Amino acid/acetate
Aspartate	0.04	0.27
Threonine	0.02	0.13
Serine	0.14	1.07
Glutamate	0.08	0.53
Glycine	0.04	0.27
Alanine	0.02	0.13
Acetate	0.15	—

TABLE II

CARBOXYL-TERMINAL ANALYSIS OF PYRUVATE KINASE AND OTHER REFERENCE PROTEINS

Results are expressed as cpm from 5 nmol of protein.

Amino acid labeled	Pyruvate kinase	Lactate dehydrogenase		Human fetal hemoglobin γ-chain
		Beef heart H_4	Rabbit muscle M_4	
Aspartate	198	218	53	28
Glutamate	353	93	98	58
Phenylalanine	28	—	1250	—
Leucine	25	2680	—	—
Histidine	274	162	150	425
Tyrosine	100	—	—	—

peptide containing glutamine or glutamate, or from pyrrolidone carboxylate. High voltage electrophoresis of this fraction yielded two spots positive to the Rydon-Smith [19] reagent. The major spot comigrated with *N*-acetylserine, contained all of the acetyl groups, and yielded equimolar quantities of serine and acetate after acid hydrolysis with only trace quantities of other amino acids. The other major spot comigrated with pyrrolidone carboxylate, and yielded glutamate upon acid hydrolysis.

In order to correct for the loss of material during the isolation procedure, tracer quantities of *N*-[^{14}C]acetylserine were added to the pronase digest of pyruvate kinase. These studies demonstrated that all of the acetyl groups present in the original pyruvate kinase preparation could be accounted for as *N*-acetylserine after correction for the loss of *N*-[^{14}C]acetylserine during the isolation procedure (25%).

Previous studies of pyruvate kinase by Cottam et al. [1] using either hydrazinolysis or carboxypeptidase procedures, failed to detect a free carboxyl-terminal residue. We have extended these studies using the tritium exchange method of Holcomb et al. [14], and the adaptation of this method described by Hsieh et al. [15]. The results of these studies are shown in Table II. In contrast to the results obtained with the standard proteins studied (M_4 and H_4 lactate dehydrogenases and human fetal hemoglobin γ-chain), the results were ambiguous. Although label was incorporated into aspartate, glutamate and histidine, no clear pattern emerged, suggesting a blocked carboxyl-terminal residue. Short term treatment of tritiated pyruvate kinase with carboxypeptidases A and B failed to release any detectable quantities of labeled amino acid. In contrast, short term treatment of tritiated human fetal hemoglobin or M_4 lactate dehydrogenase released only histidine and phenylalanine, respectively, as expected. When these proteins were subjected to long term hydrolysis with carboxypeptidases A and B, this patterns of tritiated amino acids released were similar to those obtained by acid hydrolysis.

Discussion

The present studies support the concept that rabbit muscle pyruvate kinase is composed of four highly similar, if not identical polypeptide chains. Freshly

prepared enzyme contains no detectable free amino-terminal residues, and 3.7—4 acetyl residues per mol of enzyme. The possibility of ϵ-acetyllysine or *O*-acetyl amino acid residues was eliminated, since all acetyl residues present on the intact enzyme were recovered after the pronase digest was passed over the Dowex 50 (H^+) column. Quantitative recovery experiments using *N*-[^{14}C] acetyl-L-serine demonstrate that all residues present on the intact enzyme are present at the amino termini as *N*-acetylserine.

Carboxyl-terminal studies of pyruvate kinase suggest the presence of a blocked carboxyl-terminal residue. Our studies, using the tritium exchange method of Holcomb et al. [14], support the earlier studies of Cottam et al. [1] who failed to detect free carboxyl-terminal residues using hydrazinolysis and carboxypeptidase methods. Although some label was incorporated into glutamate, aspartate and histidine in the tritium exchange studies, the label incorporated into these residues could best be explained in other ways. Non-terminal glutamate and aspartate residues incorporate label in this method [14], and pyruvate kinase contains a relatively high proportion of them [1]. The histidine labeling was similar to the background labeling of histidine noted in studies of either M_4 or H_4 lactate dehydrogenase isozymes, in which the carboxyl-terminal residues are known to be phenylalanine and leucine, respectively [20]. Control studies with a protein known to contain histidine at the carboxyl terminus, human fetal hemoglobin γ-chain [21], showed a more specific labeling pattern after both acid hydrolysis and carboxypeptidase hydrolysis methods than did pyruvate kinase. Histidine was the only tritiated amino acid released upon short term carboxypeptidase treatment of human fetal hemoglobin γ-chain, while similar treatment of pyruvate kinase failed to release any labeled amino acid indicating histidine labeling occurred at positions other than the carboxyl terminal.

The failure of tritium exchange, hydrazinolysis, and carboxypeptidase methods to detect free carboxyl-terminal residues indicate a blocked residue. The data suggest that pyruvate kinase is unusual in that both carboxyl- and amino-terminal positions are blocked.

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