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ISOLATION AND SEQUENCE DETERMINATION OF A PEPTIDE LOCATED IN OR NEAR THE ACTIVE SITE OF BOVINE MUSCLE PYRUVATE KINASE[1]

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SUMMARY

Bovine muscle pyruvate kinase was inactivated by treatment with trinitrobenzenesulfonic acid; approximately one trinitrophenyl group was incorporated per subunit. ADP or Mg-ADP decreased the rate of inactivation but Mg^{++} alone or phosphoenolpyruvate had no effect. The inactivated protein was treated with trypsin and the trinitrophenylated peptide isolated by gel filtration. Homogeneity of the isolated peptide was shown by high voltage electrophoresis and high pressure liquid chromatography. Amino acid analysis and sequence determination revealed the presence of an acidic peptide 34 amino acids long and containing ε-trinitrophenylated lysine.

Pyruvate kinase (E.C. 2.7.1.40) catalyzes the transfer of the phosphoryl group from phosphoenolpyruvate to ADP, thereby producing pyruvate and ATP. Both a monovalent and a divalent cation are required for catalytic activity, the most effective being K^{+} and Mg^{++}, respectively. The active form of the enzyme contains four subunits, and the occurrence of four phosphoenolpyruvate binding sites suggests the presence of four active sites per tetramer (1,2). Hollenberg et al. (3) found that trinitrophenylation of the ε amino group of an essential lysine in rabbit skeletal muscle pyruvate kinase resulted in the loss of ability of this enzyme to catalyze phosphoryl transfer from phosphoenolpyruvate to ADP, and similar results were obtained with the enzyme from bovine muscle (4).

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While trinitrophenylation resulted in loss of ability of the enzyme to bind ADP (5), the capability of pyruvate kinase to catalyze the phosphate-activated tritium exchange from pyruvate was retained, as was its ability to bind phosphoenolpyruvate (5) and to decarboxylate oxalacetate (6). The evidence cited above, the protective effect of ADP against inactivation, and physical studies demonstrating retention by TNP[3]-pyruvate kinase of native hydrodynamic properties and subunit structure (4) all strongly suggest that inactivation of enzymatic activity proceeds via trinitrophenylation of a group located in or at least near the ADP binding site.

In this paper we report the isolation and sequence determination of the TNP peptide from inactivated bovine muscle pyruvate kinase.

METHODS

Distilled, deionized water was used for making all solutions. Lactate dehydrogenase, trypsin, TNBS, and substrates were from Sigma Chemical Corp. Iodoacetamide was from Aldrich and sequencing chemicals were from Pierce. All other chemicals were of reagent grade. Bovine skeletal muscle pyruvate kinase was purified and assayed as described previously (2). The homogeneity was confirmed by SDS polyacrylamide gel electrophoresis (7).

For treatment with TNBS, 100-150 mg of protein in a total volume of approximately 10 ml was dialyzed against three changes, one 1 each, of 50 mM potassium phosphate, pH 8.0. The enzyme was inactivated by titration at 0° in the dark with a one percent solution of TNBS. When the enzymatic activity had decreased to about 10 percent of the original value, one ml of 1.0 M tris-HCl, pH 7.0 was added and the protein was washed by precipitation at least three times with saturated ammonium sulfate, redissolving the protein between each precipitation in 0.1 M tris-HCl, pH 7.0 containing 10 mM 2-mercaptoethanol. The final precipitate was dissolved in a minimum volume of distilled water and dialyzed against several changes of water overnight at 4°. The number of TNP-lysyl residues per mole protein was determined from the protein absorbance at 280 nm (2) and the extinction coefficient for TNP-lysine of 1.45×10^4 l/mole/cm at 346 nm (8).

Carboxymethylation was performed as described by Means and Feeney (9) except that iodoacetamide was used rather than iodoacetic acid. Approximately 100 mg of modified protein was digested with 2 mg trypsin at 30° for 3 h in 0.5 percent ammonium bicarbonate, pH 8.0. The solution was then boiled for 3 min and lyophilized.

Isolation of the TNP-peptide was accomplished by gel filtration chromatography at room temperature on a 2.5 x 100 cm column of Biogel P-6 in 0.5 percent ammonium bicarbonate. Peptide elution was monitored at 230 nm and by fluorescence production upon the addition of o-phthaldialdehyde (10). The TNP-peptide was detected by its absorbance at 346 nm.

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[3]Abbreviations used: TNP denotes trinitrophenyl, and TNBS denotes trinitrobenzenesulfonic acid.

ε-TNP-lysine was synthesized as described by Hubbard and Cardenas (4). The PTH derivative was produced by dissolving 0.6 μmole of TNP-lysine in 0.5 ml 50 percent aqueous pyridine under N_2. Twenty μl of phenyl isothiocyanate was added under N_2, the solution capped tightly, mixed vigorously, and incubated for 20 min at 50°. The solution was washed with 400 μl heptane/ethyl acetate (10:1) twice and heptane/ethyl acetate (2:1) three times. The organic phases were discarded and the final aqueous phase was dried *in vacuo*. The dried product was then converted to the PTH form by adding 40 μl 1 N HCl in methanol and incubating for 10 min at 50°. The product was dried *in vacuo*.

Sequence determinations were performed on a Beckman 890C Sequencer by the method of Edman and Begg (11) as modified by Hermodson *et al*. (12). The following sequencer programs were employed: 1.0 M Quadrol protein method (Beckman Program 122974); 0.1 M Quadrol protein method of Brauer *et al*. (13) Beckman Program 030176A); a 0.1 M Quadrol peptide method (Beckman Program 030176B), and a 0.33 M Quadrol program essentially as described by Hunkapiller and Hood (14). In some analyses, 3.0 mg of conditioned polybrene was added to allow sequencing of the C-terminal region of the peptide (14-16). Identification of PTH derivatives was accomplished by gas chromatography (17,18) and by thin layer chromatography (19). PTH-arginine was identified by the phenanthrenequinone spot test (20). PTH-ε-trinitrophenyl lysine was identified by thin layer chromatographic comparison with the synthesized compound using the following solvent systems: (a) water:acetic acid, (2:1), and (b) toluene: heptane:acetic acid, (2:1:1).

RESULTS AND DISCUSSION

Spectroscopic analysis of the modified protein at 280 and 346 nm indicated the presence of 0.75-0.80 TNP groups per subunit. Shown in Figure 1 is the elution profile for the chromatographic separation of tryptic peptides on Biogel P-6. The first peak in the elution profile contained nearly all the

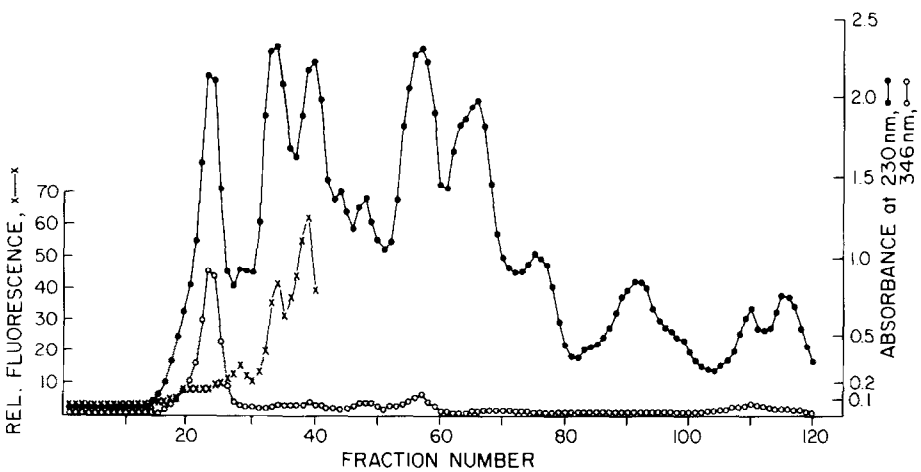

Figure 1. Elution pattern of tryptic peptides from trinitrophenylated bovine muscle pyruvate kinase as separated by chromatography on Biogel P-6. The absorbance at 230 nm is due to the presence of peptide bonds, the relative fluorescence values indicate the reactivity of eluted fractions to o-phthaldialdehyde, and the absorbance at 346 nm was used to detect the trinitrophenyl peptide. Procedures are given in the Methods section.

Table 1. Amino acid analysis of the isolated trinitrophenylated peptide. The peptide was hydrolyzed in 6 N HCl at 110° for 24 h in vacuo.

Amino Acid	Nanomoles per Sample	Mole Ratio (Based on Arginine)
S-Carboxymethyl-Cysteine	2.81	0.80
Aspartate	16.68	4.76
Threonine	2.90	0.83
Serine	6.47	1.85
Glutamate	9.68	2.76
Proline	2.79	0.79
Glycine	13.31	3.80
Alanine	19.39	5.53
Valine	9.32	2.66
Methionine	2.73	0.78
Isoleucine	3.82	1.09
Leucine	10.97	3.13
Tyrosine	2.34	0.67
Phenylalanine	trace	-
Lysine	1.33	0.38
Arginine	3.51	1.00

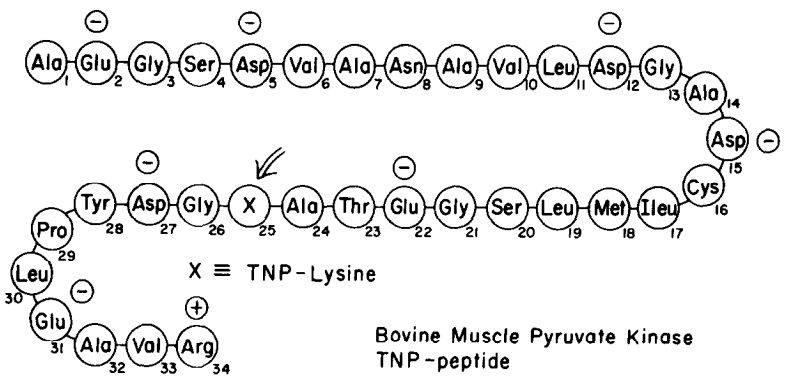

Figure 2. Sequence of trinitrophenylated peptide isolated from labeled bovine muscle pyruvate kinase. Procedures are given in the Methods section.

trinitrophenylated material. It is interesting to note that this peptide had very low reactivity with o-phthaldialdehyde. Rechromatography of the isolated TNP-peptide on Biogel P-6, high voltage electrophoretic analysis on paper, and high pressure liquid chromatographic analysis all demonstrated the peptide to be homogeneous.

Shown in Table 1 is an amino acid analysis of the isolated peptide. Amino acid ratios were normalized to those of arginine and listed in the last column of Table 1. Shown in Figure 2 is the sequence of amino acids in the peptide. The ratios of amino acids as determined from amino acid analyses

agree remarkably well with the amino acid composition predicted by sequence data. The sequence is that of a very acidic peptide, consistent with its highly anodic mobility during high voltage electrophoresis.

Several sulfhydryl alkylating reagents have been used to inactivate pyruvate kinase and some of these cysteine-containing peptides have been isolated and characterized (23-27). Amino acid sequences of cysteinyl peptides from muscle pyruvate kinases of rabbit, sturgeon, and cat seem to be somewhat similar (see references 26 and 27). However, it remains unclear whether these alkylating agents are modifying a sulfhydryl group within the active site or one that is critical for maintaining the structural integrity of the enzyme. The 6 Å X-ray crystallographic data of Stammers and Muirhead (21) with methyl mercury show a reactive sulfhydryl group in the same domain as the active site but more than 10 Å away from the binding sites. It should be noted that the peptides described in the studies cited above have little or no sequence homology with the TNP-peptide reported in this work.

Other group-specific reagents have been used to probe the active site and may shed some light on the structure of the active site. Diethyl pyrocarbonate, a reagent that reacts with histidine residues, was found by Dann and Britton (28) to inactivate rabbit muscle pyruvate kinase and by Bornmann and Hess (29) to inactivate the enzyme from yeast. Phenylglyoxal was observed by Berghauser (30) to inactivate pyruvate kinase from porcine heart, suggesting the possible involvement of an arginine. Through further sequence studies we hope to determine the structural and functional relationships of these peptides.

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