MODIFICATION OF YEAST 3-PHOSPHOGLYCERATE KINASE: ISOLATION AND SEQUENCE DETERMINATION OF A NITRATED ACTIVE-SITE PEPTIDE AND ISOLATION OF A CARBOXYL MODIFIED ACTIVE-SITE PEPTIDE

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Summary: Previous studies have shown that yeast 3-phosphoglycerate kinase is inhibited by nitration of a single tyrosine residue. Chymotryptic fragmentation of the nitrated protein followed by peptide mapping revealed approximately fifty peptides, one of which was shown to contain a nitrotyrosine residue. Isolation of this unique peptide was accomplished by gel filtration and high voltage paper electrophoresis. The sequence as established by Edman degradation and carboxypeptidase hydrolysis is: Lys-NO_2Tyr-Phe-Phe-Lys. Independent observations on the X-ray crystallographic model of yeast phosphoglycerate kinase provides supportive evindence of this sequence. Additionally, a peptide has been isolated containing an active-site carboxyl residue following modification of the enzyme with [^{14}C]methoxyamine.

Yeast 3-phosphoglycerate kinase[1] is a monomeric glycolytic enzyme of unknown primary structure. Upon nitration of PGK with tetranitromethane, there is 85% loss in enzyme activity with the modification of one residue of tyrosine and with no other residues affected (1). This paper describes the isolation and sequence determination of the peptide containing the single nitrotyrosine residue obtained after chymotryptic fragmentation. A preliminary report of this work has appeared (2). Additionally, an essential role of one carboxyl group in PGK has also been demonstrated previously by use of Woodward's reagent K (3). In an effort to locate the essential carboxyl in the structure of the enzyme, the modified protein has been reacted with [^{14}C]methoxyamine, followed by chymotryptic cleavage and peptide purification, and the composition of the active-site peptide has been determined.

Material & Methods: Yeast phosphoglycerate kinase was obtained as a crystalline suspension in 3M ammonium sulfate from Boehringer-Mannheim Corporation with a specific activity of at least 1000 units/mg at 30°C and migrated as a single band on gel electrophoresis. Glyceraldehyde 3-phosphate dehydrogenase,

[1]Yeast 3-phosphoglycerate kinase, EC 2.7.2.3, is abbreviated PGK.

3-phosphoglycerate, ATP and NADH were all purchased from Boehringer-Mannheim. Woodward's reagent K and tetranitromethane were from Aldrich and [^{14}C]methoxyamine was purchased from New England Nuclear. Chymotrypsin (TPCK-treated) and carboxypeptidases A and B were purchased from Worthington Biochemical. All other chemicals used were reagent or spectral grade. Water was double-distilled and deionized.

Enzyme Assay. The enzyme assay employed was that described previously (1).

Chymotryptic Fragmentation of Nitrated PGK. Approximately 10 μmoles of PGK nitrated as previously described (1) was suspended in 0.1 M ammonium bicarbonate, pH 8.0, and 150 μl of chymotrypsin solution (1mg/ml) was added. The mixture was incubated at 37°C for 3 hours with addition of another 300 μl aliquot of chymotrypsin half way through the incubation. The reaction was halted by the addition of 20 μl of diisopropylphosphofluoridate (2% in isopropanol).

Two-Dimensional Peptide Mapping of the Peptide Mixture Resulting from Chymotryptic Fragmentation. Approximately 30 nmole aliquots of the peptide mixture were spotted in duplicate on Whatman 3 MM chromatography paper. In the first dimension descending chromatography was performed with n-butanol:pyridine:glacial acetic acid:water (15:10:3:12) for 14 hours. The papers were allowed to air-dry for a minimum of 8 hours after which they were mounted on racks and saturated with pH 1.9 electrophoresis buffer prior to high voltage electrophoresis in the second dimension (pH 1.9, 2500 volts, 35 minutes). One of the chromatograms was dipped in 0.1% ninhydrin in collidine-ethanol which served to identify the peptides. The second chromatogram was sprayed with 50% ammonium hydroxide thereby allowing visualization of the nitrotyrosine peptide due to its yellow color at alkaline pH.

Isolation of Nitrotyrosine Peptide from the Chymotryptic Fragmentation of Nitrated PGK. The peptide mixture after chymotryptic digestion was applied to Sephadex G-25 (4.5 x 150 cm) and eluted with 0.1 M ammonium bicarbonate, pH 8.0, buffer. The absorbance of the effluent was monitered at 230 nm, 280 nm and 428 nm. Fractions containing the nitrotyrosine peptide (as evidenced by absorbance at 428 nm) were pooled and taken to dryness by rotary evaporation. The resulting peptide material was solubilized in 30% acetic acid and further fractionated on Sephadex G-10 (2 x 55 cm). Appropriate fractions were pooled, concentrated by rotary evaporation and the peptide material dissolved in 500 μl of 30% acetic acid. Aliquots were analyzed by peptide mapping (described above) and the nitrotyrosine-containing peptide was further purified by preparative pH 1.9 electrophoresis, at 2500 volts for 45 minutes. The appropriate strips were cut and the nitrotyrosine peptide eluted from the paper with 30% acetic acid.

Amino Acid Analysis. Amino acid analyses were performed by automated ion-exchange chromatography using a Beckman model 120C according to the general procedures of Spackman, Stein and Moore (4) and Moore and Stein (5).

Edman Degradation of the Nitrotyrosine Peptide. The nitrotyrosine peptide was sequentially degraded by a modification of the Edman procedure as previously described (6,7). The anilinothiazolinone was hydrolyzed directly to the free amino acid in 6 N HCl at 150°C for 16 hours and the amino acid was identified by automatic amino acid analysis.

Carboxypeptidase Hydrolysis of the Nitrotyrosine Peptide. Approximately 125 nmoles of the nitrotyrosine peptide was dried in a 5 ml conical centrifuge tube and 150 μl 0.2 M ammonium bicarbonate was added. Digestion was initiated by the addition of 50 μl of carboxypeptidase B solution. The mixture was incubated at 40°C for 60 minutes, after which a 50 μl aliquot was removed and added to glacial acetic acid to stop the reaction. After lyophilization the amino acids were identified by automatic amino acid analysis. Carboxypeptidase A was then added (50 μl) and aliquots removed from the incubation mixture

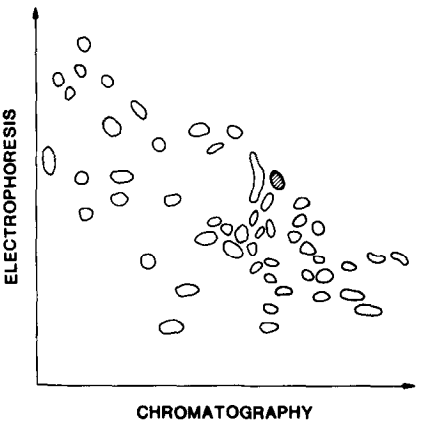

Figure 1. Two-dimensional peptide map of chymotryptic fragmentation of nitrated phosphoglycerate kinase (conditions as described in the text). The hatched spot indicates the position of the nitrotyrosine peptide localized by ammonia spraying and ninhydrin staining.

at 2, 8, 20 and 60 minutes and treated in the same manner as described above. Prior to use, carboxypeptidases A and B were diluted to 0.5 mg/ml and treated with diisopropylphosphofluoridate as previously described (8).

Preparation of Carboxyl-Modified PGK. The procedure of Brake and Weber (3) was employed to modify 200 mg of PGK with Woodward's reagent K. After exhaustive dialysis, the modified enzyme was reacted at pH 6.2, 25°C, with 1M [^{14}C] methoxyamine (250 µC) for 30 hours. The [^{14}C]-labeled enzyme (500 cpm/mg protein) was then dialyzed against double distilled water and lyophilized. Radioactivity was measured in a Beckman LS-230 liquid scintillation counter employing Bray's solution as the cocktail.

Chymotryptic Digestion of [^{14}C]PGK. Chymotryptic digestion was performed as described above using 150 mg of labeled PGK.

Gel Filtration of Chymotryptic Digest. After concentration of the digest to 5 ml, gel filtration on Sephadex G-25 (0.9 x 130 cm) was performed as described above. Fractions were monitered at 230 nm, 280 nm and by liquid scintillation counting. The main peak of radioactivity was pooled and concentrated to 9 ml.

Carboxymethyl-Cellulose Chromatography in the Presence of 8M Urea. The concentrated peak from gel filtration was made 8 M in urea and the pH was adjusted to 5.0. The sample was applied to a CM-cellulose column (2.5 x 75 cm) pre-equilibrated with 8M urea in 0.02 M ammonium acetate, pH 5.0. Elution was affected by a linear gradient of 0 to 0.18 M NaCl in the equilibration buffer. Further gradients of 0.18M to 0.36M NaCl, and 0.36M to 1.0M NaCl in equilibration buffer were employed followed by a final gradient of 1 M NaCl from pH 5.0 to 2.9. Each fraction was read at 230 nm and 280 nm and the radioactivity of each determined by liquid scintillation counting. Of the radioactivity applied to the column, 35% was irreversibly bound. The main peak of radioactive peptide, eluting in the first gradient, was pooled and passed through a Sephadex G-25 column (0.9 x 123 cm) equilibrated with 30% acetic acid. The radioactive peak was pooled and concentrated.

Preparative Thin-Layer Electrophoresis. Preparative thin-layer electrophoresis (Analtech cellulose plates) of the labeled fraction from CM-cellulose chromatography was performed at pH 1.9 (1000 volts, 40 mamp, 4°C for 30 minutes).

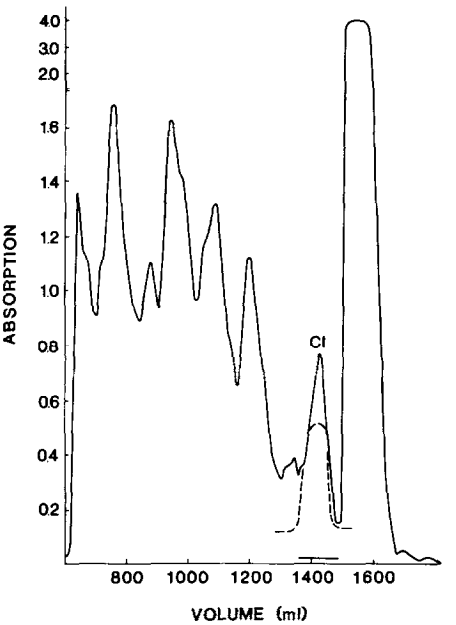

Figure 2. Gel filtration of peptide mixture obtained from chymotryptic hydrolysis of nitrated phosphoglycerate kinase on Sephadex G-25F. Solid line represents the absorption at 230 nm. Dashed line represents the single peak based on the 428/280 nm absorbance ratio. Bar indicates the fractions pooled.

Radioactive zones on the plates were detected with a Packard model 7200 strip counter. The radioactive region was scraped off and extracted with 30% acetic acid. After centrifugation the resulting supernatants were pooled and concentrated by rotary evaporation to 1 ml. Approximately 10 nmoles of peptide was applied to an Analtech cellulose thin layer plate for two-dimensional mapping: ascending chromatography in butanol:pyridine:acetic acid:water (75:50:15:60) followed by electrophoresis at pH 1.9 as described above. The plate was counted by a Packard strip counter and was then developed by use of a ninhydrin spray. Only one ninhydrin positive peptide that contained all the radioactivity was observed.

Amino Acid Analysis of Peptide. The peptide remaining after assessing purity was hydrolyzed in 6 N HCl *in vacuo* for 24 hours. The dried peptide hydrolysate was subjected to amino acid analysis on a Durrum D-500 amino acid analyzer.

RESULTS AND DISCUSSION: The two-dimensional map of the peptide mixture resulting from chymotryptic digestion of nitrated PGK is shown in Figure 1. At the alkaline pH of the ammonia solution the single nitrotyrosine peptide may be distinguished as a yellow spot.

The elution profile from Sephadex G-25 of the peptide mixture resulting from chymotryptic fragmentation of nitrated PGK is shown in Figure 2. The

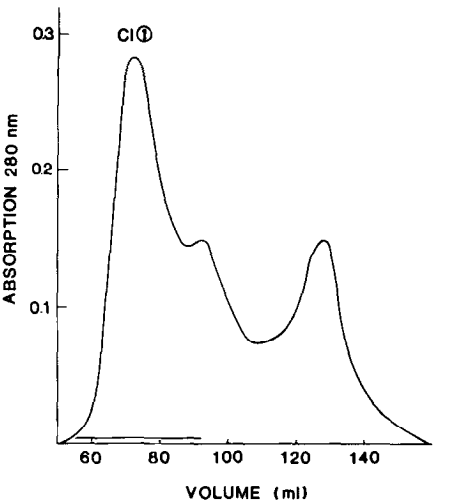

Figure 3. Gel filtration of C1 peptide fraction, obtained from Sephadex G-25F, on Sephadex G-10. Bar indicates fractions pooled.

nitrated peptide was found in fraction C1. This fraction was further resolved by gel filtration of Sephadex G-10 (Figure 3). The major peak designated C1①, was shown by analytical paper chromatography and high voltage electrophoresis to contain the nitrotyrosine peptide. Preparative paper electrophoresis was used to purify this peptide. The chromatographic and electrophoretic behavior of the purified peptide corresponded to that obtained for the nitrotyrosine-containing peptide on the two-dimensional peptide map of the original digest. The peptide was obtained in a yield of 30 % from native PGK.

The amino acid composition of this peptide was: Lys - 1.87 residues, Phenylalanine - 2.02 residues and Nitrotyrosine - 1.00 residue. Five steps of the Edman degradation gave the following sequence: Lysyl-Nitrotyrosyl-Phenylalanyl-Phenylalanyl-Lysine.

The carboxyl-terminal portion of the sequence was confirmed by carboxypeptidase hydrolysis: Carboxypeptidase B released lysine quantitatively, carboxypeptidase A plus B released lysine and increasing amounts of phenylalanine with time.

The amino acid sequence of the unique nitrotyrosine peptide is one that would not be normally expected from chymotryptic hydrolysis. However, cleavage at the carboxyl group of lysine has been noted when it occurs in a sequence of basic amino acids (8-11). The presence of a lysine-lysine bond could well explain the presence of the lysine residue at the carboxyl-terminal of the nitrotyrosine peptide. A second anomoly is the fact that the peptide contains a sequence of three aromatic amino acid residues which normally would serve as a site for cleavage by chymotrypsin. The only comment that can be made in this respect is that this rather uncommon sequence in the protein serves to cause an unusual environment that alters the expected behavior of chymotrypsin.

Subsequent to our determination of the sequence of this peptide, it was successfully fitted into the electron density map of yeast PGK obtained from X-ray diffraction studies at 3.5 Å by Bryant, et al. (12). The pentapeptide sequence is believed to start at residue 117 and is near the active site of the enzyme but on the opposite side of the ATP binding lobe from the ATP binding site in the bilobular structure of the molecule (13). It is of interest to note that the nitrotyrosine-containing peptide represents the only chemically obtained sequence which has been identified in the X-ray model to date.

Finally, the carboxyl-modified PGK possessed 36% residual activity and had 1.1 moles of carboxyl modified per mole of enzyme, as determined from incorporation of [^{14}C] label. The carboxyl-modified peptide produced by chymotryptic digestion was obtained in pure form in only 1% yield. However, the labeled peptide that was isolated was clearly the major radioactive product and under the reaction conditions employed should contain the modified essential carboxyl (3); further, this peptide was judged to be pure by thin-layer peptide mapping. The minimum amino acid composition for the labeled peptide is asp, gly, glu, ile_3, leu_2, ser. Unfortunately the low yield precluded sequence determination of the peptide at this time.

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