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IDENTIFICATION OF THE BROMOPYRUVATE-SENSITIVE GLUTAMATE WITHIN THE ACTIVE SITE OF 2-KETO-3-DEOXYGLUCONATE-6-P ALDOLASE*

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SUMMARY - Bromopyruvate inactivates 2-keto-3-deoxygluconate-6-P aldolase by a mechanism in which the reagent is incorporated by esterification. A tryptic peptide derived from inactivated enzyme has the sequence Thr-Leu-Glu*-Val-Thr-Leu-Arg. Derivatization of the γ-carboxyl of the single glutamate by bromopyruvate was confirmed by Lossen rearrangement in which the glutamate γ-ester was converted to 2,4-diamino butyrate.

Bromopyruvate is an active site-directed reagent which inactivates 2-keto-3-deoxygluconate-6-P aldolase of Pseudomonas putida, at low ionic strength, by a mechanism in which the reagent is covalently bound through ester linkage formation (1). In this paper we identify the bromopyruvate-sensitive residue as glutamic acid, and report the isolation and characterization of a unique tryptic peptide which contains that glutamate.

METHODS

Trypsin, treated with TPCK, and carboxypeptidases A and B were purchased from Worthington Biochemical Corporation. The polystyrene base cation-exchange resin DC2 (Durrum Chemical Corp.) and Sephadex G-25 (Pharmacia) were used in peptide purification procedures. Polyamide thin-layer sheets were obtained from Gallard-Schlessinger. Pyridine was distilled over ninhydrin prior to use. Reagents for sequence analysis, obtained from Pierce Chemical Co., were stored under nitrogen and used without further purification, except for trifluoroacetate

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which was distilled before use. Fluorodinitrobenzene was obtained from Pierce Chemical Co. 2,4-Diaminobutyric acid was a gift from Dr. F. C. Hartman, Biology Division, Oak Ridge National Laboratory.

The aldolase, isolated from glucose-grown P. putida, was purified to homogeneity and inactivated as previously described using [2-^{14}C]bromopyruvate under conditions where greater than 90% of the reagent was covalently bound by ester linkage (1). The stoichiometry was 1 equivalent of reagent bound per catalytic subunit inactivated, or three equivalents per mole of enzyme (2,3). The isolated, radioactive protein was denatured by addition of sodium laurylsulfate to 0.1% concentration. The [2-^{14}C]carboxyketomethyl derivative was decarboxylated to [1-^{14}C]carboxymethyl derivative by addition of H_2O_2 to 10 mM final concentration. It should be noted that free [1-^{14}C]-glycolate would result from [1-^{14}C]carboxymethyl derivatized protein by any subsequent hydrolysis of the ester linkage. The protein was recovered by chromatography on Sephadex G-25 (fine) vs. 10 mM NaCl and lyophilized.

Labeled protein was suspended in water at 20 mg/ml, heated at 100° for 2 min, and then cooled immediately in an ice bath, after which the preparation was made 50 mM with $(NH_4)HCO_3$ to maintain the pH at 8. Trypsin was then added at a molar ratio of 1:20. After two hours incubation at 25°, an equal amount of trypsin was added, and incubation was allowed to proceed two hours more. The sample was adjusted to pH 2.8 by the addition of HCl and centrifuged. About half of the radioactivity remained with the trypsin core. The supernatant from above was applied to a 0.9 x 20 cm column of Durrum DC-2 cation exchange resin maintained at 55°. Elution was achieved using a linear gradient of 0.05 M pyridine (acetate) pH 2.8 → 1 M pyridine (acetate) pH 5.0.

Sequential degradation of the purified peptide was carried out according to Edman (4). Newly exposed amino-terminal residues were determined by the method of Hartley (5) and Gray and Smith (6). Dansylated amino acid derivatives were identified by chromatography on polyamide sheets by a modification of the procedure described by Woods and Wang (7). Polyamide sheets were cut into 5 cm squares and developed in 1.5% formic acid followed by benzene-glacial acetic acid (88:10) in the second dimension. The carboxyterminal sequence was determined using carboxypeptidases B and A.

RESULTS

Purification and composition. The soluble peptides from a tryptic digest of 250 nmoles of ^{14}C-labelled enzyme were subjected to cation exchange chromatography as shown in Fig 1. Although 5-10% of the radioactivity is found early in the elution profile, 60% of the applied label appears as a major peak about one-quarter of the way through the total elution profile. The early peak, as the free acid, is volatile and thus is presumed to be [1-^{14}C]glycolate resulting from deesterification which occurs during handling. Amino acid analysis of acid hydrolysates of the major peak showed it to be impure. The peak was pooled, concentrated by lyophilization, redissolved in water, and purified

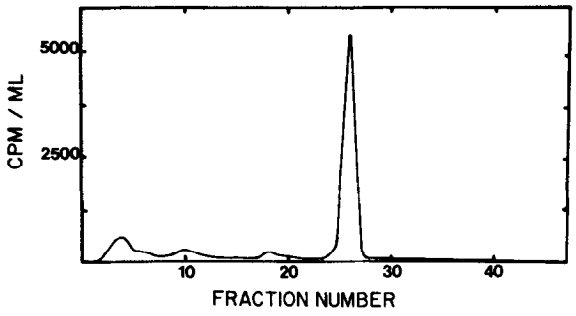

FIG. 1. Cation exchange chromatography on Durrum DC-2 resin, of the tryptic hydrolysate of [1-^{14}C]carboxymethyl 2-keto-3-deoxygluconate-6-P aldolase derived from [2-^{14}C]bromopyruvate inactivated enzyme followed by oxidative decarboxylation. 250 nanomoles of enzyme were digested with trypsin at a molar ratio of 1:20 at 25° and pH 8 as described in Methods. Elution was achieved using a linear gradient of 0.05 M pyridine (acetate) pH 2.8 → 1 M pyridine (acetate) pH 5.0, at a flow rate of 10 ml per hr. The 0.9 x 20 cm resin bed was maintained at 55°.

further by gel filtration on Sephadex G-25 (2.5 x 30 cm) in 10 mM sodium acetate-50 mM acetic acid. The eluted radioactivity (50% recovery) was pooled lyophilized, dissolved in water, and subjected to further gel filtration in the same column. The result of the second gel filtration step is presented in Fig. 2 where coincidence of radioactivity and peptide bond-containing material (A_{218}) is shown. Amino acid analysis of a hydrolysate of this material yielded a heptapeptide comprised of Arg, Thr_2, Glu, Val, Leu_2.

Sequence analysis. Digestion with carboxypeptidase B released arginine, confirming its carboxyterminal position in the peptide. As shown in Table I, subsequent incubation of the peptide with carboxypeptidase A released, sequentially, only an additional three residues, Leu, Thr, and Val. Three sequential Edman degradations were performed on another 10 nmoles of the peptide, and the newly released amino termini were identified as Thr, Leu, Glu, and Val, respectively. Conditions required for dansylation led to saponification of

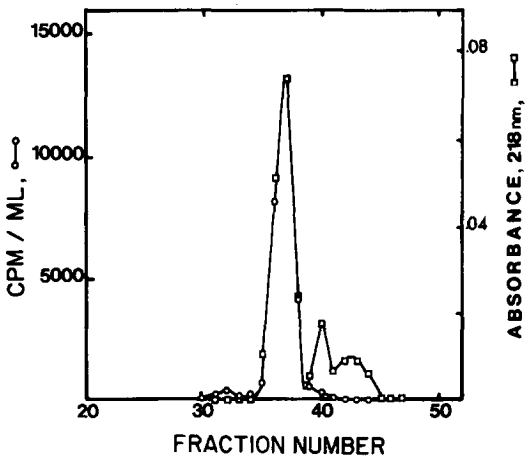

FIG. 2. Second chromatography of the tryptic peptide of [1-^{14}C]-carboxymethyl 2-keto-3-deoxygluconate-6-P aldolase derived from [2-^{14}C]-bromopyruvate inactivated enzyme followed by oxidative decarboxylation. 250 nanomoles of enzyme were chromatographed on a 2.5 x 30 cm bed of Sephadex G-25 (fine) using 10 mM sodium acetate-50 mM acetic acid as the eluant. Radioactivity was determined by liquid scintillation counting, and peptide bond containing material was visualized at A_{218}.

γ-carboxymethyl-glutamate ester so that only the dansylated free amino acid was detected. Therefore, the complete sequence of the peptide can be deduced to be:

$$\underset{\rightarrow}{\text{Thr}}\text{-}\underset{\rightarrow}{\text{Leu}}\text{-}\underset{\rightarrow}{\text{Glu*}}\text{-}\underset{\leftrightarrow}{\text{Val}}\text{-}\underset{\leftarrow}{\text{Thr}}\text{-}\underset{\leftarrow}{\text{Leu}}\text{-}\underset{\leftarrow}{\text{Arg}}$$

Glutamate derivatization. The identity of the γ-carboxyl of glutamate as the site of derivatization by bromopyruvate (glycolate) was confirmed by Lossen rearrangement (8,9). In this procedure, the glycolate ester is displaced by hydroxylamine, and the peptide hydroxyamate is alkylated using fluorodinitrobenzene. After extraction of excess reagent with ether, the dinitrobenzyl hydroxamyl peptide is heated at alkaline pH to effect the final conversion of a γ-glutamyl ester to 2,4-diaminobutyrate (8). The hydroxyamino-peptide (75 nmoles) was recovered by chromatography on Sephadex G-25. A fluorescamine-

Table I

Carboxypeptidase Digestion of the Radioactively Labeled Tryptic Peptide Derived from Bromopyruvate-inactivated Aldolase

	Amino acid (nanomoles)				
Time	Thr	Val	Leu	Arg	Glu
0	-	-	-	1	0
15	0.39	0	1.14	1.0	0
30	0.45	0	1.11	1.0	0
60	0.43	0.40	0.77	1.0	0
120	1.10	0.90	1.15	1.0	0

Thr-Leu-Glu*-Val-Thr-Leu-Arg

The peptide (20 nmoles) was treated with 1 μgm of carboxypeptidase B and preincubated 15 min at 37°. Then 0.1 μgm of carboxypeptidase A was added at 0 time and timed aliquots, representing 2 nanomoles, were diluted into pH 2.2 citrate buffer. Half of the sample was then studied using a Durrum D-500 Amino Acid Analyzer.

positive peak was found to elute at that position where the radioactive peptide eluted prior to derivatization. The radioactivity eluted at a position expected for a small molecular weight species and was assumed to be glycolate. Following Lossen rearrangement, amino acid analysis of an acid hydrolysate of the peptide showed loss of glutamate, and its replacement by 0.8 residue equivalent of 2,4-diaminobutyrate. This confirms that derivatization of the peptide by radioactive bromopyruvate (glycolate) is at the γ-carboxyl of the single glutamate.

DISCUSSION

The results of these experiments provide unequivocal evidence that bromopyruvate inactivates 2-keto-3-deoxygluconate-6-P aldolase by a mechanism in which the γ-carboxyl of a protein-bound glutamate attacks C-3 of the analog, displacing the bromide, to form an ester linkage. A tryptic peptide isolated

from inactivated enzyme has the sequence,

Thr-Leu-Glu*-Val-Thr-Leu-Arg.

Since previous studies have established that the bromopyruvate is active site-directed (1,3,10,11), the bromopyruvate-sensitive glutamate must lie within the catalytic site. Further, this glutamate may well provide that basic group involved in the protiotropic activation(s), occurring during catalysis, thought to be mediated by this aldolase (12).

It is noteworthy that the amino acid sequence reported in this study is not contained within that of a cyanogen bromide-generated 50 amino acid peptide containing the Schiff's base-forming lysine which is 10 residues removed from the amino terminus (13). Consequently, the glutamic acid residue participating in proton activation(s) during catalysis could be far removed in linear sequence from the lysine residue which interacts with substrate to generate the electron sink. It will be of interest, in future work, to determine whether this is true as well as locate the position of this glutamate, with respect to the Schiff's base lysine, within the folding of the catalytic subunit (14).

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