IDENTIFICATION OF THE HISTIDYL RESIDUE OF RABBIT MUSCLE ALDOLASE ALKYLATED BY N-BROMOACETYLETHANOLAMINE PHOSPHATE*

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Received January 14,1974

SUMMARY: The site-specific reagent N-bromoacetylethanolamine phosphate has previously been shown to inactivate aldolase by alkylation of an essential histidyl residue. To determine the position of this residue in the primary sequence, we have isolated the labeled peptides in tryptic digests of aldolase after its inactivation by ^{14}C reagent. Two peptides, which contain the histidine derivative and have apparently identical amino acid compositions, are obtained. These peptides lack Arg and Lys, and thus appear to be derived from the C-terminal portion of the aldolase molecule. This assumption is confirmed by carboxypeptidase digestion of the modified aldolase and the purified peptides. Thus, the histidyl residue alkylated by the reagent is the one adjacent to the penultimate residue. The finding that two peptides arise from the C-terminal region is consistent with the previous finding that in the α subunits the penultimate residue is Asn and in the ß subunits is Asp.

BrAcNHEtOP[1] has recently been synthesized, and tested as an affinity labeling reagent for fructose bisphosphate aldolases (1). Although the reagent is not specific for a single residue, inactivation of aldolase from both rabbit and rat muscle correlates with the alkylation of a histidyl residue. Since a variety of data indicated that the histidyl residue is probably at or near the active site, and may even be involved in the catalytic process, it becomes of interest to identify the residue's position within the primary structure of aldolase. The methods by which this identification has been made are described in this report.

MATERIALS AND METHODS

Fructose-1,6-bisphosphate, NADH, glycylglycine, Pipes, and a mixture of glycerophosphate dehydrogenase and triose phosphate isomerase were purchased from the Sigma Chemical Co. Rabbit muscle aldolase with a specific activity of 7.5 units/mg

*Research sponsored by the United States Atomic Energy Commission under contract with the Union Carbide Corporation.

[1]The abbreviations used are: BrAcNHEtOP, N-bromoacetylethanolamine phosphate; Pipes, piperazine-N,N'-bis(2-ethanesulfonic acid).

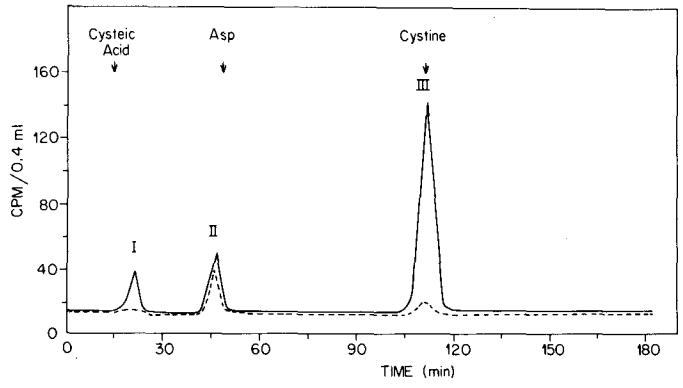

Figure 1. — Chromatographic profiles of radioactive components present in acid hydrolysates of aldolase after treatment with [^{14}C]BrAcNHEtOP in the absence (——) and presence (- - - -) of butanediol bisphosphate, as described in Materials and Methods. An amount of hydrolysate equivalent to about 3 mg of protein was applied to the long column of the amino acid analyzer. Fractions of the effluent were collected at 3-min intervals, and 0.4-ml aliquots of these fractions were counted. The absorbance at 570 nm is not shown, but the elution positions of several amino acids are indicated.

was a product of Boehringer-Mannheim Corp. Trypsin treated with L-(tosylamido-2-phenyl)ethyl chloromethyl ketone and carboxypeptidase A treated with diisopropyl fluorophosphate were obtained from the Worthington Biochemical Corp. Butanediol bisphosphate and ^{14}C-labeled BrAcNHEtOP (185,000 cpm/μmole) were synthesized by published procedures (1, 2). Protein concentrations, aldolase activity, radioactivity, and amino acid compositions were determined by methods described and cited previously (1).

The chemically modified aldolase to be used in characterization studies was prepared by differential labeling. To a solution of aldolase (200 mg) in 20 ml of 0.05 M Pipes–1 mM EDTA (pH 6.5) were added 64 mg (5 mM) of the competitive inhibitor butanediol bisphosphate and 54 mg (10 mM) of unlabeled BrAcNHEtOP. This solution was incubated at room temperature for 4 days, at which time the enzymic activity had decreased to 74% of its initial value. Virtually no activity was lost from a control solution. The modified enzyme was dialyzed successively against 0.05 M sodium phosphate, 0.01 M sodium acetate, and 0.05 M Pipes. All three buffers contained 1 mM EDTA and were at pH 6.5. After dialysis the protein concentration was 9.3 mg/ml. A 15-ml portion of the dialyzed enzyme was incubated with 4.05 mg (1 mM) of [^{14}C]BrAcNHEtOP. A 5-ml portion was incubated with 16 mg (5 mM) of butanediol bisphosphate and 1.35 mg (1 mM) of the labeled reagent. The remainder of the dia-

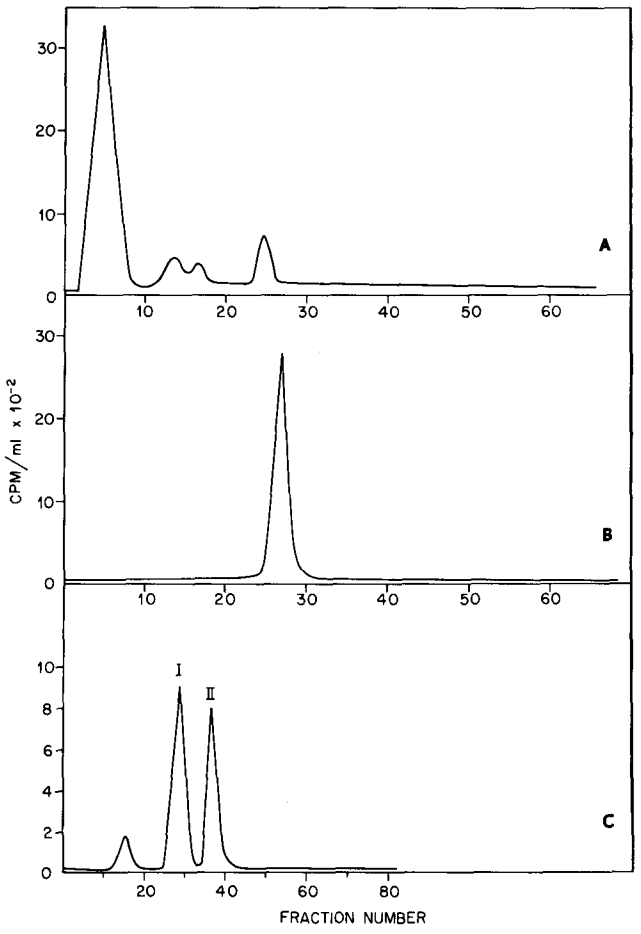

Figure 2. — Purification of labeled peptides from a tryptic digest of aldolase after treatment with [^{14}C]BrAcNHEtOP as described in Materials and Methods. (A) Ion-exchange chromatography of the digest (equivalent to about 20 mg of protein) on a 1 X 25 cm column of Bio-Rad AG 50W-X2. The column was eluted with a linear gradient composed of 150 ml each of 0.2 M pyridine–4.6 M acetic acid (pH 3.1) and 2.0 M pyridine–2.4 M acetic acid (pH 5.0). (B) Gel filtration of the lyophilized radioactive pool (tubes 4-6) from the ion-exchange column on a 1.8 X 220 cm column of Bio-Gel P-4. The column was equilibrated and eluted with 0.01 M ammonium bicarbonate (pH 8.0). (C) Ion-exchange chromatography of the lyophilized radioactive pool (tubes 26-28) from the gel filtration column on a 1 X 25 cm column of Whatman DEAE cellulose (DE-52). The column was eluted with a linear gradient (200 ml each) of ammonium bicarbonate (pH 8.1, 0.02–0.04 M). Tubes 28-30 and 36-38 were pooled separately, lyophilized, and used in the characterization studies.

lyzed enzyme served as a control. After the solutions had remained at room temperature for 48 hours, the amounts of aldolase activity remaining relative to that of the control (which was unaltered) were 97% and 33% for the samples treated in the presence and

absence of the protector, respectively. The samples were dialyzed exhaustively as above, except that in the last buffer change 0.1 M sodium bicarbonate (pH 8.0) was used instead of Pipes.

RESULTS AND DISCUSSION

Cysteinyl, methionyl, and histidyl side-chains are alkylated during the course of inactivation of aldolase by BrAcNHEtOP (1). However, butanediol bisphosphate, which protects against inactivation, prevents primarily the alkylation of histidine. Thus, inactivation is a consequence of histidine modification (1). To simplify the isolation of the peptide containing the essential histidyl residue that is preferentially alkylated, we first subjected aldolase to high concentrations of unlabeled reagent in the presence of a protector so as to label all sites not involved in the inactivation process. After removal of the protective agent and excess reagent, the enzyme was retreated with ^{14}C-labeled reagent in the absence and presence of protector. The inactivated aldolase thus obtained contained 1.4 molar equivalents of reagent per mole of enzyme, whereas the protected sample contained only 0.15. Acid hydrolysates of the protein samples were examined on the amino acid analyzer to verify that the label was associated with histidine derivatives. Cleavage of the amide linkage in the protein-bound reagent moiety results in the release of all modified residues as their carboxymethyl derivatives, whose elution positions from the analyzer are well established (3, 4). The radioactive profiles obtained are shown in Figure 1. The major radioactive species arising from the inactive aldolase elutes at the position of cystine and is therefore identified as 3-CM-His (peak III). Much smaller amounts of CM-Cys (peak II) and a presumed decomposition product of CM-Met (peak I) are also present. The only clearly identifiable radioactive component found in hydrolysates of aldolase that was labeled in the presence of protector is CM-Cys.

The differentially labeled aldolase was carboxymethylated and digested with trypsin by usual procedures (5). The resulting ^{14}C-labeled peptides were isolated by column chromatography (Figure 2). DEAE-cellulose chromatography, the last step in the purification, resolved two labeled peptides that were present in about equal amounts. Peptide mapping (data not shown) indicated that the two peptides were essentially pure; their amino acid compositions are shown in Table I. As anticipated, both peptides contain one residue that yields 3-CM-His. The two peptides have seemingly identical

TABLE I

Amino acid compositions of labeled peptides

Amino acid	Peak I		Peak II		C-terminal peptide (ref. 6)
	μMoles found	No. of residues	μMoles found	No. of residues	
Trp	None*	0	None*	0	0
Lys	Trace	0	None	0	0
His	None	0	None	0	1
Arg	Trace	0	Trace	0	0
CM-Cys	None	0	None	0	0
Asx	0.011	1.0	0.014	1.4	1
Thr	0.011	1.0	0.010	1.0	1
Ser	0.038	3.6	0.038	3.8	4
Glx	0.021	2.0	0.029	2.9	2
Pro	0.010	1.0	0.012	1.2	1
Gly	0.023	2.2	0.024	2.4	2
Ala	0.050	4.8	0.049	4.9	5
3-CM-His†	0.011	1.0‡	0.010	1.0‡	0
Val	Trace	0	0.003	0.3	0
Met	None	0	None	0	0
Ile	0.009	0.9	0.012	1.2	1
Leu	0.011	1.0	0.011	1.1	1
Tyr	0.018	1.7	0.020	2.0	2
Phe	0.011	1.0	0.012	1.2	1

*Based on lack of absorbancy at 280 nm.

†All of the radioactivity applied to the analyzer was recovered in the 3-CM-His peak.

‡Arbitrarily set to 1.0.

compositions and do not contain either Lys or Arg. The absence of both Lys and Arg in peptides derived from a tryptic digest strongly suggests that the peptides are derived from the C-terminal region of the protein. Consistent with this possibility is that the amino acid composition of the peptides agrees with the C-terminal sequence of aldolase (6).

Two C-terminal peptides also result from tryptic digestion of native rabbit muscle

TABLE II

Amino acids released from modified aldolase and labeled peptides during digestion with carboxypeptidase A

Amino acid	Moles released per mole of protein or peptide				
	Aldolase*			Labeled peptides†	
	Native	Inactivated‡	Protected‡	Peak I	Peak II
His	1.4	0.12	0.9	—	—
Ser + Asn§	2.1	1.9	2.0	0.5	—
Ala	2.9	3.1	2.8	1.0	0.4
Ile	1.6	1.4	1.4	0.2	—
Leu	2.0	1.7	2.0	0.3	—
Tyr	2.8	2.7	2.8	1.0	1.0
Phe	1.1	1.0	0.9	Trace	—

*Aldolase (4 mg/ml) in 0.2 M N-ethylmorpholinium acetate (pH 8.0) was treated with carboxypeptidase A (0.1 mg/ml) at 25°C for 24 hours. After the solutions were acidified to pH 2.0 and centrifuged, aliquots were subjected to amino acid analysis.

†The peptides (0.02 μmoles) in 0.2 ml of 0.2 M N-ethylmorpholinium acetate (pH 8.0) were treated with 0.02 mg of carboxypeptidase A at 25°C for 6 hours. The solutions were acidified to pH 2.0 and aliquots were subjected to amino acid analysis.

‡Native aldolase was alkylated with BrAcNHEtOP (10 mM) in the absence and presence of butanediol bisphosphate (5 mM) under the same conditions described in Materials and Methods. The inactivated sample retained 5% of its initial activity; the protected sample retained 80% of its initial activity.

§Not resolved by the chromatographic conditions used.

aldolase (7). This is a consequence of aldolase being composed of two kinds of subunits, designated α and ß, which differ at the position three residues removed from the C-terminus. Since in the α subunit this residue is Asn and in the ß it is Asp (7), two chromatographically different peptides arise that cannot be distinguished by amino acid analysis of acid hydrolysates.

To confirm that the labeled peptides were derived from the C-terminal portion of aldolase, the chemically modified enzyme and the purified peptides were digested with carboxypeptidase A. The C-terminal sequence of aldolase determined in part by car-

boxypeptidase digestion (7, 8) is –Phe–Leu–Ile–Ser–Asx–His–Ala–Tyr. If the histidyl residue in this segment is the one alkylated by BrAcNHEtOP, less histidine should be liberated, by carboxypeptidase digestion, from the inactivated aldolase than from the native enzyme or the enzyme protected from inactivation by butanediol bisphosphate. These anticipated results are obtained (Table II). Digestion of the purified peptide that eluted first from the DEAE column (the less acidic of the two peptides and therefore presumably the one containing Asn) released the same amino acids as are released from native aldolase (Table II). The conditions used did not result in complete digestion, but the relative amounts of amino acids released agree with the known C-terminal sequence of aldolase. The other purified peptide (the more acidic one, that presumably contains Asp) yielded only Tyr and Ala upon digestion with carboxypeptidase (Table II) as is the case for the ß subunit of aldolase (7). We conclude that BrAcNHEtOP inactivates aldolase by the preferential alkylation of the histidyl residue closest to the C-terminal end.

Since alkylation of a histidyl residue in aldolase results in loss of fructose bisphosphate cleavage activity without appreciable change in transaldolase activity, we speculated that the imidazolium side-chain might be the group that catalyzes proton removal from, and addition to, the C-3 carbon atom of the enzyme-bound dihydroxyacetone phosphate moeity (1). Photoinactivation studies had previously implicated a histidyl residue in this role (9, 10). With the realization that the alkylated histidyl residue is close to the C-terminus, one must consider the possibility that the alterations in catalytic properties are a result of conformational changes. Removal of the C-terminal tyrosyl residues of aldolase (11) leads to similar alterations in enzymic activity (12), so perhaps any chemical change at the C-terminal region destroys the enzyme's ability to effect the proton transfer step in question, even though the acid-base group that catalyzes this step is not modified.

REFERENCES

1. Hartman, F. C., Suh, B., Welch, M. H., and Barker, R. (1973) J. Biol. Chem. 248, 8233-8239.
2. Hartman, F. C., and Barker, R. (1965) Biochemistry 4, 1068-1075.
3. Gurd, F. R. N. (1972) Methods Enzymol. 25B, 424-438.
4. Goren, H. J., Glick, D. M., and Barnard, E. A. (1968) Arch. Biochem. Biophys. 126, 607-623.
5. Salter, D. W., Norton, I. L., and Hartman, F. C. (1973) Biochemistry 12, 1-5.
6. Lai, C. Y., and Horecker, B. L. (1972) Essays Biochem. 8, 149-178.

7. Lai, C. Y., Chen, C., and Horecker, B. L. (1970) Biochem. Biophys. Res. Commun. 40, 461-468.

8. Winstead, J. A., and Wold, F. (1964) J. Biol. Chem. 239, 4212-4216.

9. Hoffee, P., Lai, C. Y., Pugh, E. L., and Horecker, B. L. (1967) Proc. Nat. Acad. Sci. U.S.A. 57, 107-113.

10. Davis, L. C., Brox, L. W., Gracy, R. W., Ribereau-Gayon, G., and Horecker, B. L. (1970) Arch. Biochem. Biophys. 140, 215-222.

11. Drechsler, E. R., Boyer, P. D., and Kowalsky, A. G. (1959) J. Biol. Chem. 234, 2627-2634.

12. Rose, I. A., and O'Connell, E. L. (1969) J. Biol. Chem. 244, 126-134.