Inactivation of Class I Fructose Diphosphate Aldolases by the Substrate Analog N-Bromoacetylethanolamine Phosphate*

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SUMMARY

N-Bromoacetylethanolamine phosphate, prepared by the bromoacetylation of ethanolamine phosphate, has been tested as an active site-specific reagent for rabbit and rat muscle fructose diphosphate aldolases. The reagent inactivates both enzymes, and inactivation is prevented by substrates or competitive inhibitors. Loss of activity is pseudo-first order until the later stages of inactivation, and a rate-saturation effect is observed as the reagent concentration is increased. The stoichiometry of the reaction has been determined with 14C-labeled reagent. Inactivated enzyme contains 2 to 2.5 molar eq of reagent per mole of aldolase subunit, whereas the enzyme modified in the presence of a competitive inhibitor contains only 1 to 1.5 eq of reagent. Thus, alterations in the catalytic properties of aldolase that accompany its modification apparently reflect the alkylation of a single, essential residue. Characterization of the protein derivatives reveals that the preservation of enzymatic activity is due primarily to the protection of a histidyl residue. This observation supports earlier evidence for the involvement of a histidyl residue in aldolase activity.

There are two major types of fructose 1,6-diphosphate aldolases, which have been designated by Rutter (2) as Class I and Class II. Class I aldolases form Schiff-base intermediates with their ketonic substrates (3) but have no requirement for divalent metal ions; Class II aldolases do not form Schiff bases (2) but contain tightly bound Zn$^{2+}$, which is functional in the catalytic process (4). The most extensively characterized Class I aldolase is the enzyme from rabbit muscle, and yeast aldolase is the most thoroughly studied Class II enzyme.

Many chemical and kinetic studies have provided a fairly detailed conceptual picture of the reaction pathway followed by the aldolase-catalyzed reaction (for a recent review see Reference 5). The classical experiments of Horecker (3) on the borohydride-induced inactivation of aldolase in the presence of dihydroxyacetone phosphate led to the identification of an essential amino group that reacts with the carbonyl of the substrate, with the elimination of water, to form a Schiff base. Oxygen exchange studies using fructose-1,6-P$_2$ labeled in the carbonyl position with $^{18}$O are consistent with the Schiff base being an obligatory intermediate (6). Protonation of the Schiff base labilizes a C-3 hydrogen atom as a proton, which is presumably accepted by an acid-base group of the enzyme, giving rise to a reactive carbanion. The existence of a carbanion intermediate was inferred from isotopic exchange studies (7-9) and verified by detection with tetranitromethane (10). The carbanion then condenses with the carbonyl of 1,6-dihydroxyacetone-P, and after condensation another acid-base group has been invoked in the transfer of a proton to the carbonyl oxygen (which becomes the C-4 hydroxy of fructose-1,6-P$_2$). Fructose-1,6-P$_2$ is released from the enzyme upon hydrolysis of the Schiff base. That the reaction pathway does require a sequential addition of substrates has been demonstrated (11, 12).

Despite the wealth of information about the reaction pathway, the identity of the enzyme's functional groups that are catalytically essential has not been rigorously established. The only unequivocal identification is that of the Schiff base-forming amino group, which can be covalently tagged with dihydroxyacetone phosphate. This has permitted comparative studies of primary structure around one active site residue, and the high degree of homology observed provides convincing evidence for the functional importance of this region of the aldolase molecule (9). In contrast, the groups involved in proton addition to and removal from the substrate have not been definitively identified, although histidyl and cysteinyl side chains have been implicated (13). We have attempted to use affinity labeling to identify essential residues in aldolase, having previously designed chloroacetyl ethanolamine phosphate; DCC, dicyclohexylcarbodiimide; Pipes, piperazine-$N,N'$-bis(2-ethanesulfonic acid).
**EXPERIMENTAL PROCEDURE**

**Materials**

Fructose-1,6-P$_2$, dihydroxyacetone phosphate, glycolglycine, ethanolamine phosphate, NADH, Pipes, and a mixture of glycerophosphate dehydrogenase and triose phosphate isomerase were purchased from Sigma Chemical Co. DCC and N-hydroxysuccinimide were products of Aldrich Chemical Co. Other materials and vendors were as follows: rabbit muscle aldolase, Boehringer-Mannheim Corp.; biological grade guanidine hydrochloride, Schwarz-Mann; and $[^{14}]$Bromoacetic acid, Amersham-Searle Corp. Rat aldolase was isolated by the method of Suh and Barker (20). Butanediol diphosphate was synthesized according to the procedure of Hartman and Barker (21). Both rabbit and rat muscle aldolases were stored as ammonium sulfate suspensions. Before use in chemical modification studies, these suspensions of aldolase were dialyzed thoroughly against 50 mM Pipes-1 mM EDTA (pH 6.5).

**Methods**

**Protein Assays**—Aldolase concentrations were calculated from the absorbances at 280 nm, using an A$_{280}$ value of 9.38 for the rabbit enzyme (22) and 9.39 for the rat enzyme (20). A molecular weight of 160,000 was assumed for both enzymes (20, 23).

**Aldolase Assays**—Aldolase was assayed by the method of Blobstein and Rutter (24) with a Beckman Acta V recording spectrophotometer. Each assay solution contained 0.05 mM glycolglycine (pH 7.5), 1 mM fructose-1,6-P$_2$, 0.15 mM NADH, and 28 $\mu$g of glyceraldehyde phosphate dehydrogenase-triose phosphate isomerase in a total volume of 3 ml. At 24$^\circ$, the specific activity of the commercial rabbit aldolase was 11.5 units per mg. Pure rabbit muscle aldolase has specific activities varying from 14 to 18 units per mg (25). The specific activity of the rat aldolase was 11.0 units per mg; the preparation used had been stored for 1 year and when first isolated had a specific activity of 13.5 units per mg.

**Radioactivity Assays**—Radioactivity was measured in a Packard liquid scintillation spectrometer. Protein solutions (0.05 to 0.2 ml) were first dissolved in 1 ml of Beckman Bio-Solv (HIBS-3), and then 10 ml of scintillation fluid, composed of 4.6 g of 2,5-diphenyloxazole (PPO) and 115 mg of 1,4-bis-[2-(5-phenyloxazolyl)]benzene (POPOP) in 1 liter of toluene-ethanol (4:3, v/v), were added. Aliquots (0.4 ml) of fractions collected from the amino acid analyzer were added directly to the scintillation solution without prior dissolution in the solubilizer.

**Amino Acid Analyses**—When applicable, samples were subjected to performic acid oxidation before being hydrolyzed, according to the procedure described by Hirs (26). Proteins were hydrolyzed with 6 $\times$ HCl in evacuated, sealed tubes at 110$^\circ$ for 24 hours. The hydrolysates were concentrated to dryness on a rotary evaporator and analyzed with a Beckman 120C amino acid analyzer by the method of Spackman et al. (27). If quantitation of tryptophan was desired, the hydrolysis was carried out in the presence of thioglycolic acid (28).

**Synthesis of BrAc†HETOP**—A solution of ethanolamine phosphate (1.41 g, 10 mmoles) in 10 ml of water was adjusted to pH 5.0 with 5 $\times$ lithium hydroxide. Bromoacetyl bromide (2.2 ml, 25 mmoles) was added dropwise, while the pH was kept at 5.0 by the addition of 5 $\times$ lithium hydroxide. The reaction mixture was then concentrated to dryness, giving a white powder that was washed several times with ether followed by ethanol. The residual BrAc†HETOP (2.4 g; 88%) appeared homogeneous by thin layer chromatography on cellulose-coated plastic sheets (Brinkmann MN-Polygram Cel 300) with an R$_f$ of 0.55 in butanol acetic acid water (7:2:5).

$$\text{C}_4H_8\text{BrLi}_3\text{NO}_3\text{P}$$
Calculated: C 17.54, H 2.58, Br 29.18, N 5.11, P 11.31
Found: C 17.30, H 2.61, Br 29.04, N 4.94, P 11.12

**Synthesis of N-Hydroxysuccinimide [14C]Bromoacetate** (29)—N-Hydroxysuccinimide (83 mg, 0.72 mmoles) was dissolved, with heating, in 3 ml of ethyl acetate. The solution was cooled to room temperature; upon addition of [14C]bromoacetic acid (100 mg, 0.72 mmoles; 200 $\mu$Ci per mmoles) and DCC (150 mg, 0.72 mmoles) an immediate exothermic reaction occurred. The reaction mixture was left at room temperature for 2 hours, and the insoluble dyeeholoxylene was then removed by filtration. The filtrate was concentrated to dryness, and the crystalline residue was recrystallized from 5 ml of isopropyl alcohol to give 135 mg (0.57 mmoles, 79%) of the desired compound, m.p. 114 to 116$^\circ$.

**Synthesis of [$^{14}$C]BrAc†HETOP**—The procedure described in an adaptation of the method used to synthesize peptides from N-hydroxysuccinimide esters of amino acids (30, 31). To an aqueous solution (0.55 ml) of ethanolamine phosphate (50 mg, 0.30 mmoles) were added 0.055 ml (0.4 mmoles) of triethylamine, 0.75 ml of tetrahydrofuran, and 115 mg (0.48 mmoles) of N-hydroxysuccinimide [14C]Bromoacetoate. Two layers formed initially, but after the mixture was stirred for about 1 min, a homogeneous solution resulted. The reaction mixture was left at room temperature for 1 hour and then cooled to 4$^\circ$ in an ice bath, at which time 300 mg (1 mmoles) of barium bromide in 3 ml of methanol was added. The phosphate ester was precipitated by the addition of lutidin (0.15 ml, 1.3 mmoles) and ethanol (3 ml). The precipitate was collected by centrifugation, washed three times with 3-ml portions of methanol, and dried in a desiccator. After dissolution in 1 ml of water, the material was freed of barium ions by the addition of a slurry of 6 g of Dowex 50 (H$^+$) in methanol (3 ml). The resin was removed by filtration, and the filtrate was adjusted to pH 8.0 with 1 $\times$ lithium hydroxide. The solvent was removed under a stream of dry nitrogen, and the residue was washed three times with 3-ml portions of ethanol and dried to give 70 mg (71%) of the desired compound with a specific radioactivity of 185,000 cpm per $\mu$mole.

This material was chromatographically indistinguishable from unlabeled BrAc†HETOP prepared from ethanolamine phosphate and bromoacetyl bromide.

**RESULTS**

**Kinetics of Inactivation and Demonstration of Substrate Protection**—Upon incubation of aldolase from both rabbit and rat muscle with BrAc†HETOP, a time-dependent, irreversible inactivation occurs. Loss of activity approximates pseudo-first order kinetics until about 30% of the initial activity remains (Fig. 1). From 70 to 90% inactivation, the rate at which enzymatic activity is lost becomes progressively slower with obvious deviation from first order kinetics. Dihydroxyacetone phosphate and the competitive inhibitor 1,4-butanediol diphosphate (21) afford substantial protection against inactivation (Fig. 1). The modified aldolase retains substantial transaldolase activity and is also partially active with fructose-1-P as substrate (Table I).

From inspection of the inactivation rates with 1 and 10 mM reagent (Fig. 1), it is clear that the rates are not directly proportional to the concentration of reagent; therefore, the reaction of BrAc†HETOP with aldolase is not a true bimolecular reac-
Figure 1. Inactivation of rabbit and rat muscle aldolase by BrAcNHEtOP at room temperature. The reaction mixtures, buffered with 0.05 M Pipes-1 mM EDTA (pH 6.5), contained rabbit muscle aldolase (0.5 mg per ml) with no additions (△), 1 mM reagent (□), 10 mM reagent (■), 1 mM reagent plus 2.5 mM dihydroxyacetone phosphate (▲), or 1 mM reagent plus 2 mM butanediol diphosphate (▲). Periodically, 0.01-ml aliquots were withdrawn and assayed for activity as described under "Methods." Rat muscle aldolase (0.5 mg per ml) was treated under the same conditions with no additions (A), 1 mM reagent (O), 10 mM reagent (●), 1 mM reagent plus 2.5 mM dihydroxyacetone phosphate (▲), or 1 mM reagent plus 2 mM butanediol diphosphate (▲). Periodically, 0.01-ml aliquots were withdrawn and assayed for activity as described under "Methods."

Table I

Fructose-1,6-P\(_2\), fructose-1-P and transaldolase activity of rabbit muscle aldolase after treatment with BrAcNHEtOP

Aldolase at 5 mg per ml was incubated with 10 mM BrAcNHEtOP as described in the legend to Fig. 1. Periodically, 0.01-ml aliquots were diluted to 1 ml with 0.05 M glycylglycine (pH 7.5); 0.1-ml aliquots of these solutions were assayed in the usual fashion (see "Methods") and in assay medium containing 25 mM acetaldehyde. Activity toward fructose-1-P was determined with 0.01-ml aliquots of the undiluted reaction mixture; fructose-1-P was present in the assay mixture at a concentration of 0.01 M.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activity % of initial</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fructose-1,6-P(_2)</td>
<td>100</td>
</tr>
<tr>
<td>Fructose-1-P</td>
<td>100</td>
</tr>
<tr>
<td>Fructose-1,6-P(_2) + acetaldheyde</td>
<td>100</td>
</tr>
</tbody>
</table>

Inactivation. If the reagent initially interacts noncovalently with the active site to form a complex that is obligatory to the formation of covalently modified inactive enzyme,

\[
E + I \xrightarrow{k_{-1}} EI \xrightarrow{k_2} E - I
\]

the rate of inactivation will approach a maximum value as the inhibitor concentration approaches infinity. A linear expression

\[
\tau = \frac{1}{[I]} \left(\frac{TK_{\text{inact}}}{T} + T\right)
\]

for the rate of enzyme inactivation has been derived (32, 33), in which \(K_{\text{inact}} = (k_{-1} + k_2)/k_1\) and comparable to \(K_m\) in the Michaelis-Menten expression. \(T\) is the minimal inactivation half-time, and \(\tau\) is the observed inactivation half-time at inhibitor concentration \([I]\). In Fig. 2, plots of \(\tau\) versus the reciprocal reagent concentration are illustrated for the inactivation of rabbit and rat aldolase. Clearly, extrapolation to infinite reagent concentration gives positive values for the half-times of inactivation. If the reactions were bi-no-molecular, the \(\tau\)-intercept would be zero. The calculated values for \(K_{\text{inact}}\) are 0.87 mM and 1.27 mM for rabbit and rat aldolase, respectively.

Extent of Incorporation. The amounts of BrAcNHEtOP incorporated into aldolases during their inactivation have been determined by use of the \(^3\)C-labeled reagent (Table II). Although more than 1 molar eq of reagent per mole of subunit is incorporated during inactivation, the difference in the amount of reagent associated with the inactivated aldolase as compared to aldolase that was protected with butanediol diphosphate is about 1 residue per subunit molecule. The degrees of protection observed are about the same, whether we use values calculated by extrapolation to 100% inactivation (in cases where 1 mM reagent was used and less than complete inactivation was achieved) or values obtained directly (in cases where 10 mM reagent was used and 96 to 99% inactivation was achieved).

Nature of Modified Residues. Since alkylation of a protein by BrAcNHEtOP introduces a substituted carbamylmethyl moiety, upon total acid hydrolysis of the protein derivative the alkylated residues are released as carboxymethyl derivatives. This provides a convenient method for characterizing the aldolase derivatives, because carboxymethyl amino acids can be identified and quantitated with the amino acid analyzer (34, 35). The elution profiles of radioactivity present in acid hydrolysates of aldolase inactivated by BrAcNHEtOP and aldolase treated with the reagent in the presence of butanediol diphosphate are shown in Fig. 3. On the basis of elution position, Peak V is 3-carboxymethylhistidine, Peak III is carboxymethylcysteine, and Peak IV (a minor component) is either 1-carboxymethylhistidine or carboxymethylhomocysteine (a degradation product of carboxymethylmethionine), which are not resolved by the standard conditions used for the long column of the amino acid analyzer (35). The identity of Peak V as CM-His is unquestionable, since it is the only known CM-derivative that elutes in coincidence with cystine (35). This coincidence does not preclude quantitating the 3-CM-His on the basis of ninhydrin reactivity, because cystine is absent in samples hydrolyzed in the presence of 0.1 M mercaptoethanol. That Peak III is indeed CM-Cys is supported by the slightly lower —SH contents of the modified aldolases as compared to native (data not shown), observed by direct titra-
TABLE II
Covalent incorporation of BrAcNHEtOP into aldolase

Aldolase at 5 mg per ml was incubated with the 14C-labeled reagent in the absence and presence of butanediol diphosphate as described in the legend to Fig. 1. Subsequent to modification, the enzyme solutions were dialyzed at 4° against 0.05 M sodium phosphate (pH 6.5) followed by 0.01 M NaCl. After dialysis, the solutions were assayed for protein and radioactivity as described under "Methods."

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Reagent concentration</th>
<th>Butanediol diphosphate concentration</th>
<th>Enzyme activity</th>
<th>Molar equivalents per mole of subunit</th>
<th>No. of residues protected per subunit</th>
<th>Treatment following removal of unbound reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mM</td>
<td>mM</td>
<td>% remaining</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit</td>
<td>1</td>
<td>0</td>
<td>16</td>
<td>1.90</td>
<td>1.90</td>
<td>1.21</td>
</tr>
<tr>
<td>Rabbit</td>
<td>1</td>
<td>5</td>
<td>95</td>
<td>0.88</td>
<td>0.85</td>
<td>1.24</td>
</tr>
<tr>
<td>Rabbit</td>
<td>10</td>
<td>0</td>
<td>2</td>
<td>3.54</td>
<td>2.30</td>
<td>2.10</td>
</tr>
<tr>
<td>Rabbit</td>
<td>10</td>
<td>5</td>
<td>90</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>1</td>
<td>0</td>
<td>35</td>
<td>0.84</td>
<td>0.84</td>
<td>0.74</td>
</tr>
<tr>
<td>Rat</td>
<td>1</td>
<td>5</td>
<td>93</td>
<td>0.36</td>
<td>0.36</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>10</td>
<td>0</td>
<td>3</td>
<td>2.93</td>
<td>2.93</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>10</td>
<td>5</td>
<td>88</td>
<td>2.10</td>
<td>2.10</td>
<td></td>
</tr>
</tbody>
</table>

a Aldolase is tetrameric with a subunit molecular weight of 40,000 (23, 47).
b The sample was incubated for 18 hours at room temperature with 0.1 N sodium hydroxide and then dialyzed exhaustively against 0.01 M NaCl.
c The sample was incubated for 18 hours at room temperature with 8 M guanidine hydrochloride—1 M hydroxylamine (pH 8.0) and then dialyzed exhaustively against 0.01 M NaCl.

*Fig. 3. Chromatographic profiles of radioactive components present in acid hydrolysates of rabbit (A and B) and rat (C) aldolase after treatment with [14C]BrAcNHEtOP. The aldolase samples used in these experiments were the same ones described in Table II, which were prepared with 1 mM reagent either in the absence (---) or presence (----) of butanediol diphosphate. An amount of hydrolysate equivalent to about 1 mg of protein was applied to the long column of the amino acid analyzer. Fractions of the effluent, after passing through the calorimeter, were collected at 3-min intervals and counted as described under "Methods." The absorbance at 570 nm is not shown, but the ion of unhydrolyzed protein with Ellman's reagent (36). Also, Peak III is susceptible to oxidation as is CM-Cys (37). Thus, at least a portion of Peak II may be the sulfoxide of CM-Cys, because if reducing agent is not present during the hydrolysis (the data in Fig. 3A were obtained with samples that were hydrolyzed in the presence of mercaptoethanol), Peak III is diminished with a corresponding increase in Peak II (Fig. 3B). Peak I, which is ninhydrin-negative, has been tentatively identified as a degradation product of CM-Met (see below). A fraction of Peak II may also have arisen from CM-Met. The numbers of histidyl, cysteinyl, and methionyl residues alkylated by BrAcNHEtOP are given in Table III. These values and the data presented in Fig. 3 show that the major difference between the inactivated and protected aldolase samples is the extent of histidyl alkylation. The numbers of residues per molecule of rabbit muscle aldolase that butanediol diphosphate protects from alkylation by BrAcNHEtOP are 2.2 histidyl, 0.3 cysteinyl, and 1.1 methionyl.

Identity of Peak I—As mentioned above, Peak I (Fig. 3) appears to be a degradation product of a carboxymethyl derivative, as it is ninhydrin-negative. Derivatives which are known to be degraded by the conditions used to hydrolyze proteins are those of glutamic acid, aspartic acid (esters), and methionine (sulfonium salt). Esters can be eliminated from consideration, as neither hydroxylamine nor alkali resulted in the removal of covalently bound reagent from the modified aldolase (Table II). Carboxymethylsulfonium salts of methionine decompose in hot acid to methionine, homoserine, and carboxymethylhomocysteine (35, 38). Byproducts, which contain the carboxymethyl moiety, are glycic acid, S-methyl-thioglycolic acid, and chloroacetic

elution positions of certain amino acids are indicated by arrows at the top of the figure. The profiles shown in B are from hydrolysates of the same samples whose profiles are shown in A, except that 0.1 M β-mercaptoethanol was not included during the hydrolysis.
TABLE III
Quantities of carboxymethyl amino acids in hydrolysates of rabbit muscle aldolase after alkylation with BrAcNHEtOP

The values given were calculated from data obtained with the same samples whose chromatographic profiles are shown in Fig. 3A. Of the radioactivity associated with the protein samples, about 95% was recovered in hydrolysates after concentration. Virtually all of the radioactivity applied to the long column of the amino acid analyzer was recovered in the collected fractions.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Methionine</th>
<th>Methionine sulfone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native aldolase</td>
<td>0</td>
<td>2.6</td>
</tr>
<tr>
<td>Aldolase inactivated with BrAcNHEtOP (15% active)</td>
<td>0.3</td>
<td>1.8</td>
</tr>
<tr>
<td>Aldolase treated with BrAcNHEtOP in presence of butanediol diphosphate</td>
<td>0.1</td>
<td>2.0</td>
</tr>
</tbody>
</table>

* Peaks I and II are not CM-Met but rather are decomposition products of CM-Met (see text).
* These values are not extrapolated to 100% inactivation.

acid (35). These compounds elute from the long column close to the front, as does Peak I. Since CM-Met is resistant to oxidation by performic acid (38), any methionine found in hydrolysates of the aldolase derivatives after oxidation must have arisen from alkylated methionyl residues. The results of such analyses are shown in Table IV. The only consideration inconsistent with alkylation of methionyl residues by BrAcNHEtOP is that CM-homocysteine is the major product formed from CM-Met in ribonuclease (35, 38) whereas the aldolase derivatives that we have characterized give rise to little, if any, CM-homocysteine. A possible explanation is that the ratio of products is altered by the substituent on the carboxymethyl side chain.

**DISCUSSION**

Although BrAcNHEtOP is not a particularly close analog of aldolase substrates, we believed it would have an affinity for the enzyme's active site in view of the variety of anionic substances that competitively inhibit aldolase. Most inorganic anions inhibit aldolase, divalent ions being much more effective than monovalent ions (39). Monophosphate esters that are competitive inhibitors include acetol phosphate (12), L-glyceraldehyde 3-phosphate (12), anhydropentitol monophosphates (21), and aryl monophosphates (20). Comparisons of molecular models of BrAcNHEtOP and the furanose ring forms of fructose-1,6-P<sub>2</sub> show that, irrespective of whether the phosphate group of the reagent binds at the site for the C-1 or C-6 phosphate group of fructose-1,6-P<sub>2</sub>, the reactive portion of the reagent could approach the position anticipated to be occupied by the groups on the enzyme that catalyze the proton-transfer steps.

If a compound is a true active site-directed reagent (an affinity labeling agent) with an absolute specificity for a catalytically essential residue, the following criteria will be met. (a) Total inactivation will occur. (b) With a large molar excess of reagent, the loss of activity will be pseudo-first order. (c) At infinite reagent concentration, the rate constant of inactivation will be finite. (d) The reagent will be a competitive inhibitor of the enzyme as well as an irreversible inactivator. (e) Substrates and competitive inhibitors will retard the rate of inactivation. (f) One molar equivalent of reagent per mole of catalytic subunit will be covalently incorporated into the enzyme as it is inactivated. (g) Due to conservation of active site structure, corresponding enzymes from diverse species will be inactivated by the reagent. In the case of inactivation of aldolase by BrAcNHEtOP, these criteria are only partially fulfilled.

Alkylation of aldolase by BrAcNHEtOP does not result in total abolishment of enzymatic activity. The most rigorous inactivation conditions that have been used (10 mM reagent for 3 days, Fig. 1) result in a 99% loss of fructose-1,6-P<sub>2</sub> cleavage activity (Fig. 1) and a significant loss of fructose-1-P cleavage activity (Table I). However, the modified enzyme retains most of its transaldolase activity, as can be demonstrated by the inclusion of acetaldehyde in the assay mixture (Table I). Thus, the aldolase derivative can still bind fructose-1,6-P<sub>2</sub> but is apparently deficient in its ability to catalyze proton abstraction from dihydroxyacetone phosphate and protonation of the enzyme-bound carbamion of dihydroxyacetone phosphate. Similar alterations in the catalytic properties of aldolase result from photoinactivation (40), carboxypeptidase treatment (12, 41), and modification with L-glyceraldehyde 3-phosphate (12, 42).

The loss of aldolase activity is not pseudo-first order throughout the course of inactivation. This is probably a reflection of the fact that BrAcNHEtOP is not specific for a single amino acid residue of aldolase, and it could indicate that there is more than one site which upon modification results in changes in catalytic properties. In spite of the lack of specificity and the complex kinetics of inactivation, a rate-saturation effect can be demonstrated (Fig. 2); therefore, a dissociable enzyme-reagent complex may be a precursor to inactivated enzyme. BrAcNHEtOP competitively inhibits aldolase (data not shown), but the $K_i$ (4.5 to 7 mm) is significantly larger than the dissociation constant (1 mm) obtained from the inactivation data. We cannot offer a definitive explanation for this apparent contradiction, but possibly different complexes are detected by the two methods. Aldolase contains two distinct anion-binding sites (43), which...
have markedly different affinities for monophosphate esters.\(^2\) Thus, 2 molecules of the inhibitor may be bound to the active site, in which case the observed values of \(K_i\) and \(K_{inact}\) would depend on the range of inhibitor concentration used.

The incorporation of BrAcNHEtOP into aldolase is not stoichiometric; however, butanediol diphosphate prevents inactivation of the enzyme by protecting 1 residue per molecule of subunit (Table II). Therefore, it was of particular interest to identify and quantitate the protected residues—a relatively simple task, since the alkylated residues are converted during hydrolysis to their corresponding carboxymethyl derivatives. Hydrolysates of the aldolase derivatives contain CM-Cys, 3-CM-His, and a presumed degradation product of CM-Met (Fig. 3). Since there is much less (2 to 2.5 residues per tetrameric aldolase molecule) 3-CM-His in samples modified in the presence of butanediol diphosphate than in inactivated samples, we believe that the inactivation of aldolase by BrAcNHEtOP results primarily from the alklylation of a single, essential histidyl residue. The extent of histidine protection (Table III) does not, however, account for the total number of residues protected on the basis of incorporation data (Table II), and clearly some protection of cysteine and methionine is observed (Tables III and IV). The number of cysteiny1 residues protected is too small for the alkylation of such residues to be a significant factor in the inactivation. Alkylation of methionyl residues may, however, contribute to the inactivation process. It is conceivable that the reagent binds at both the site for dihydroxyacetone phosphate and the site for glyceraldehyde 3-phosphate. Binding at one site could lead to alkylation of a histidyl residue, while binding at the other site could lead to alkylation of a methionyl residue. We have not yet been able to determine whether in a given molecule the modification of one of these residues prevents the modification of the other.

It is obvious that the reaction of BrAcNHEtOP with aldolase is a far from perfect example of affinity labeling. Nevertheless, the reagent may prove to be a useful probe for defining structural regions in aldolases that are important in catalysis. BrAcNHEtOP is the first reagent reported to inactivate aldolase by alkylating histidyl and methionyl residues. On the basis of photoinactivation studies, Horecker and colleagues (40) postulate that an imidazole side chain is the group that transfers proton to and from the C-3 carbon atom of dihydroxyacetone phosphate. This postulate is based primarily on the observation that photoinactivation of aldolase, like the alkylation reported here, does not destroy the enzyme’s transaldolase activity. If the same histidyl residue is the target of both oxidation and alkylation by BrAcNHEtOP, it may well be catalytically essential. The striking similarities, in all aspects investigated, between the reactions of BrAcNHEtOP with rabbit and with rat muscle aldolases leads further support to the concept that the reactive histidyl residue plays a crucial role in the catalytic process. The present study also provides the first example of substrate protection of histidyl and methionyl residues in aldolase. Perhaps the best evidence, albeit indirect, that BrAcNHEtOP is an active-site-directed reagent is that bromoacetic acid and isodesamido, whose chemical reactivities should be similar to those of our reagent, inactivate aldolase by alkylating sulfhydryl groups (44, 45).

We are aware of the many potential pitfalls in the interpretation of chemical modification studies (46). The resolution of whether the histidyl residue labeled by BrAcNHEtOP is catalytically functional or even in the vicinity of the active site or whether it is distant from the active site and protected from modification as a result of substrate-induced conformational changes will at least require comparative sequence studies to determine the degree of homology adjacent to the reactive histidyl residue. In this connection, we are currently isolating the labeled peptides from proteolytic digests of modified rabbit and rat muscle aldolases. We are also attempting to determine the location of the reagent moiety with respect to the binding sites for dihydroxyacetone phosphate and glyceraldehyde 3-phosphate.

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Inactivation of Class I Fructose Diphosphate Aldolases by the Substrate Analog N-Bromoacetylethanolamine Phosphate
Fred C. Hartman, Byungse Suh, Mary H. Welch and Robert Barker


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