AFFINITY LABELING OF YEAST ALDOLASE BY HALOACETOL PHOSPHATES*

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SUMMARY—Under mild conditions, chloro- and bromoacetol phosphate, reactive analogs of dihydroxyacetone phosphate, inactivate yeast fructose diphosphate aldolase by alkylation of about one SH group per molecule of catalytic subunit. Protein side-chains other than SH are not modified, since the amount of reagent incorporated is equivalent to the loss of SH groups. The observed stoichiometry, the kinetics of inactivation, and substrate protection data suggest that the SH group alkylated by these reagents is in the active-site region of this class II aldolase.

Class I (Schiff-base-forming) and class II (metal-requiring) Fru-1,6-P_2 aldolases have a common mechanism involving the C-3 carbanion of DHAP† (1-3), and they may also be structurally related in that their amino acid compositions and subunit sizes are quite similar (4). The active site of rabbit muscle aldolase (the prototype of class I enzymes) has been partially characterized (5-9), whereas the active site of yeast aldolase (the prototype of class II enzymes) has not been characterized except for the finding that enzyme-bound Zn^{++} is essential for catalysis (10,11). To probe further the active sites of Fru-1,6-P_2 aldolases, "affinity labeling" (12-14) with haloacetol phosphates is being attempted.

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†Abbreviation used: DHAP, dihydroxyacetone phosphate.

The synthesis of these reagents and their reactions with rabbit muscle aldolase have been reported previously (15, 16). In this communication, we report experiments suggesting that chloro- and bromoacetol phosphate selectively modify the active site of yeast aldolase.

MATERIALS AND METHODS

Yeast aldolase was prepared according to the procedure of Rutter et al. (17) with the modification described by Kobes et al. (10). The enzymic activities of these preparations, as assayed according to Richards and Rutter (18), were 40-90 μmoles of Fru-1,6-P_2 cleaved per min per mg of protein. All enzyme solutions were desalted by passage over a Sephadex G-25 column equilibrated with 0.05 M glycylglycine at pH 7.5 or by extensive dialysis against the same buffer. The molecular weight of yeast aldolase was assumed to be 80,000 and the ϵ at 280 nm was taken to be 1.02 (4). The following materials were purchased from the Sigma Chemical Co.: NADH, DHAP, Fru-1,6-P_2, glycerol phosphate dehydrogenase, and triose phosphate isomerase. Tritium-labeled $NaBH_4$ (specific radioactivity, 200 Ci/mole) was obtained from the New England Nuclear Corporation. Haloacetol phosphates were prepared and assayed as previously described (15).

RESULTS

Inactivation of Yeast Aldolase by Haloacetol Phosphates

Figure 1 shows the time-course of inactivation of aldolase by chloro- and bromoacetol phosphate. The initial 50% loss of activity approximates pseudo first-order kinetics; and although the reaction rate subsequently decreases, inactivation is virtually complete within 4 hr. The second-order rate constants for the reactions determined from the initial inactivation rates with 0.1 mM reagents are 2.7 and 56 M^{-1} sec^{-1} for the chloro and bromo reagents, respectively. If the initial half-life of inactivation (τ) by chloroacetol phosphate is plotted as a

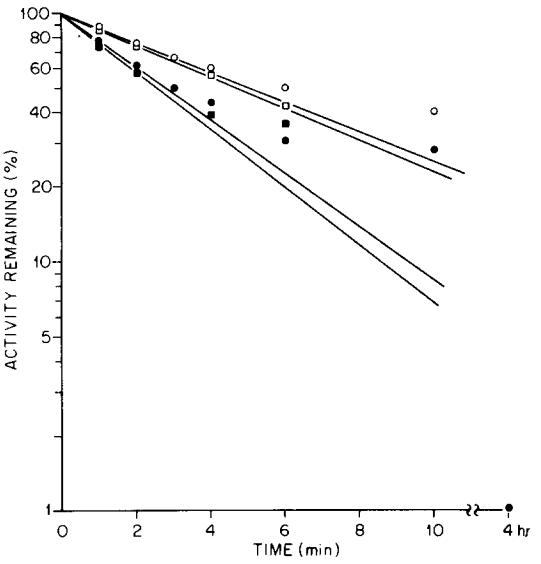

FIG. 1. Kinetics of yeast aldolase inactivation by chloro- and bromoacetol phosphate at room temperature. Reaction mixtures, buffered with 0.05 M glycylglycine (pH 7.5), contained aldolase (0.1 mg/ml) with chloroacetol phosphate at 1 mM (O) and 4 mM (●) or aldolase (0.17 mg/ml) with bromoacetol phosphate at 0.04 mM (□) and 0.08 mM (■). The buffer used for the experiments with chloroacetol phosphate also contained 0.1 M potassium acetate. Periodically, aliquots of the reaction mixture were diluted into assay buffer, containing 0.01 M ß-mercaptoethanol to decompose excess reagent, and assayed.

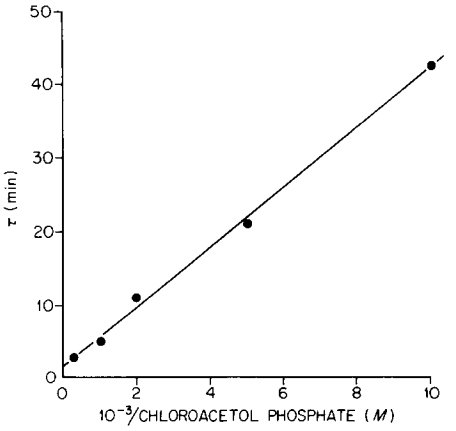

FIG. 2. Inactivation half-life (τ) determined from initial rates as a function of the reciprocal of chloroacetol phosphate concentration. The conditions were as described in the legend of Fig. 1.

function of reciprocal reagent concentration, τ approaches a limiting, finite value (Fig. 2), indicative of the formation of a dissociable enzyme-inhibitor complex

before covalent bond formation (19, 20). The apparent dissociation constant of the complex (the reagent concentration giving the half-maximal rate of inactivation) is 2.5×10^{-3} M, a value close to the K_M (2×10^{-3} M) for DHAP (21); the first-order rate constant calculated from the minimal half-life of inactivation (the y-intercept) is 6.6×10^{-3} sec^{-1}. (For the derivations of the equations used to calculate these constants see references 19 and 20).

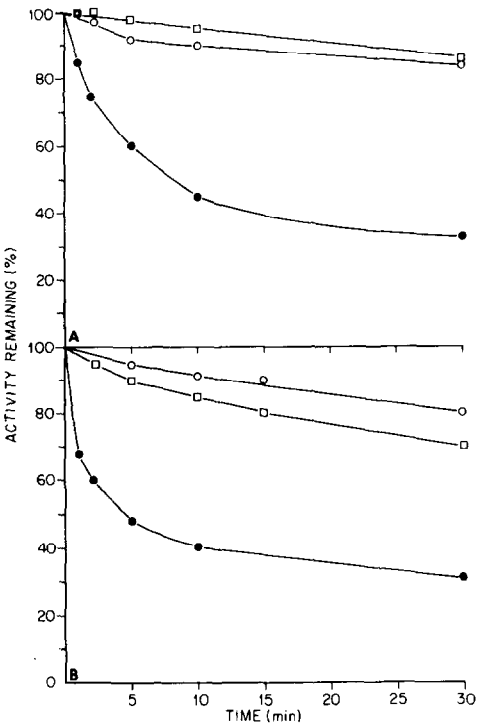

FIG. 3. Protective effects of Fru-1,6-P_2 and DHAP on the inactivation of aldolase by haloacetol phosphates. (A) Reaction mixtures contained aldolase (0.1 mg/ml) and 1 mM chloroacetol phosphate with either no other additions (●), 5 mM Fru-1,6-P_2 (○), or 5 mM DHAP (□). (B) Reaction mixtures contained aldolase (0.1 mg/ml) and 0.1 mM bromoacetol phosphate with either no other additions (●), 5 mM Fru-1,6-P_2 (○), or 5 mM DHAP (□). Other conditions were as described in the legend of Fig. 1.

In Fig. 3 are illustrated the protective effects of substrates against the inactivation of aldolase by the two reagents.

Incorporation of Haloacetol Phosphates into Aldolase and Loss of Enzyme Sulfhydryl Groups

TABLE I

Extent of Reagent Incorporation and SH Modification upon Inactivation of Aldolase by Haloacetol Phosphates[a]

Reagent	Moles ^{32}P/ mole aldolase[b]	Moles SH/mole aldolase[c]		
		Native	Inactivated	No. modified
[^{32}P] Chloroacetol phosphate	1.6	8.3	6.8	1.5
[^{32}P] Bromoacetol phosphate	2.5	8.1	5.3	2.8

[a]The modified aldolase samples used for these determinations contained only 5—10% of the initial enzymic activity. The values reported were obtained by extrapolation to 100% inactivation.

[b]Incorporation of [^{32}P]haloacetol phosphates into aldolase was determined by measuring radioactivity in aliquots that had been removed from the reaction mixture and dialyzed exhaustively.

[c]SH groups were measured in the presence of urea or guanidine by Ellman's procedure (24).

As shown in Table I, aldolase inactivated with chloro- and bromoacetol [^{32}P] phosphate contains 1.5 and 2.5 moles of ^{32}P per mole of protein, respectively. Dialysis against 8 M guanidine hydrochloride does not decrease the amount of protein-bound radioactivity, thereby demonstrating the covalent nature of the protein-reagent bond.

The only demonstrable reactivity of chloro- and bromoacetol phosphate toward model compounds is alkylation of SH groups (15). Thus, to determine whether aldolase SH groups are modified by these reagents, SH measurements were performed on the native and inactivated enzymes. The results of these assays (Table I) show that aldolase SH groups are modified and that the number modified is within experimental error of ^{32}P incorporation. The values obtained

for the unmodified enzyme are in good agreement with those obtained by amino acid analyses and titrations with p-hydroxymercuribenzoate (4, 22).

The following experiment was performed to verify that the equivalence of reagent incorporation and SH loss was not fortuitous (e.g., SH loss could be due to an oxidative process), but actually represented alkylation of aldolase SH groups. The enzyme, inactivated with chloroacetol phosphate, was treated with [^{3}H]$NaBH_4$, which reduces the carbonyl of the incorporated reagent to a hydroxyl group, thereby introducing a tritium label. This protein derivative was then subjected to acid hydrolysis followed by chromatography on the

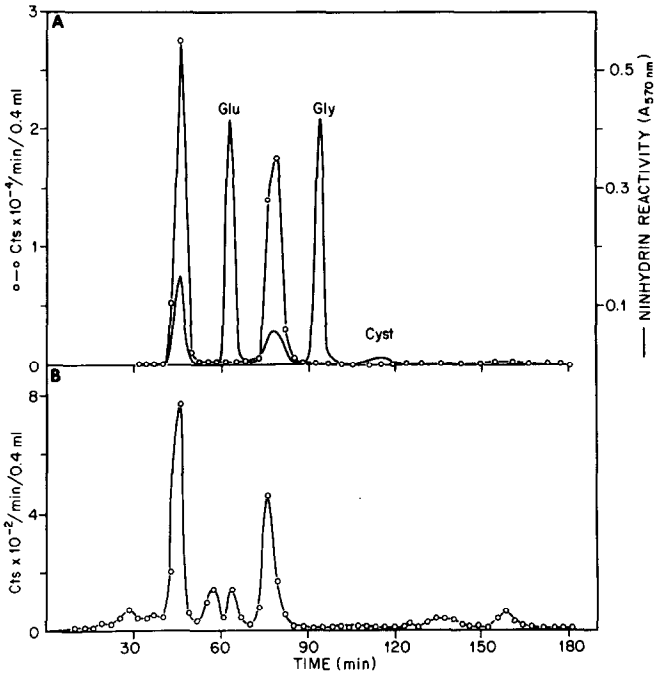

FIG. 4. Chromatography on a Beckman Model 120C amino acid analyzer of acid hydrolysates of glutathione and yeast aldolase after their modification with chloroacetol phosphate followed by reduction with [^{3}H]$NaBH_4$. (A) Glutathione (0.01 M) in 1 ml of 0.1 M $NaHCO_3$ was treated with chloroacetol phosphate (0.011 M) for 1 hr, after which [^{3}H]$NaBH_4$ (0.4 mg) was added to the reaction mixture. After 30 min, the solution was acidified and concentrated several times from water, and a portion was hydrolyzed (6 N HCl at 110° for 21 hr). (B) Yeast aldolase (3.2 mg) in 2 ml of 0.05 M glycylglycine (pH 7.5) was incubated with 1 mM chloroacetol phosphate for 2 hr, after which only 15% of the initial activity remained. The sample was then reduced as described for glutathione, dialyzed exhaustively, and a portion was hydrolyzed.

automatic amino acid analyzer. The major peaks of radioactivity coincided with those found in a hydrolysate of glutathione subsequent to its alkylation with chloroacetol phosphate and reduction with [^{3}H]$NaBH_4$ (Fig. 4).

DISCUSSION

Inactivation of yeast aldolase by chloro- and bromoacetol phosphate is due solely to modification of SH groups. This conclusion is based on (a) the equivalence of the extents of reagent incorporation and loss of SH groups and (b) demonstration that most of the protein-bound reagent is present as an S-alkyl cysteine derivative. The presence of two major radioactive peaks in acid hydrolysates of both glutathione and yeast aldolase subsequent to their alkylation with chloroacetol phosphate and reduction with [^{3}H]$NaBH_4$ is probably due to instability of the cysteine derivative to the hydrolytic conditions.

Yeast aldolase contains two subunits, each of 40,000 daltons, and two active sites per molecule (4, 23). Thus, the observed stoichiometry of incorporation (about 2 moles of reagent per mole of aldolase) is consistent with a selective modification of the active site. The greater incorporation of bromoacetol phosphate as compared to the chloro compound may reflect some random modification of SH groups. Actually, the incorporation of less than one molar equivalent of chloroacetol phosphate per subunit has been observed repeatedly. One explanation is that the aldolase used in these studies, although essentially homogeneous as judged by disc gel electrophoresis, has a somewhat lower specific activity than reported previously.

The rate saturation effect observed with chloroacetol phosphate (Fig. 2) suggests that the reagent possesses an affinity for the DHAP binding site and thus forms a dissociable complex with that site before covalent reaction. Also, the similarity of the dissociation constant of the enzyme-reagent complex to the

K_M of DHAP suggests that the reagent is interacting with the substrate binding site. The protection against inactivation afforded by DHAP and Fru-1,6-P_2 is further evidence of active-site modification. It is unlikely that alkylation of a single SH per molecule of aldolase subunit induces gross conformational changes near the active site, because the zinc content of the inactivated enzyme is identical to that of the native enzyme.

A previous investigation by Ingram (22) concerning the inactivation of yeast aldolase by p-hydroxymercuribenzoate and 5,5'-dithiobis(2-nitrobenzoic acid) suggested that SH groups may be essential for catalysis; however, in his studies selective modification of a single SH was not achieved. Although definitive proof is still lacking, our experiments support the possibility that an SH group in yeast aldolase is involved in catalysis.

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