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Haloacetol Phosphates. Selective Alkylation of Sulfhydryl Groups of Rabbit Muscle Aldolase by Chloroacetol Phosphate†

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ABSTRACT: Chloroacetol phosphate (3-chloro-1-hydroxy-2-propanone phosphate), a reagent structurally similar to the substrate dihydroxyacetone phosphate, has been examined as a potential active-site-specific reagent of rabbit muscle aldolase. At neutral pH, the reagent is a competitive inhibitor of the enzyme (K_I = 0.6 mM). Based on its ability to quench aldolase fluorescence and to protect the enzyme against trypsin inactivation, chloroacetol phosphate appears to bind at the dihydroxyacetone phosphate site. However, Schiff-base formation between reagent and enzyme (also, indicative of interaction with the dihydroxyacetone phosphate binding site) was not detected by the criterion of inactivation with borohydride. At pH 10.0, the reagent irreversibly inactivates the enzyme. Several observations suggest that chloroacetol phosphate inactivates aldolase by the preferential alkylation of one sulfhydryl group per catalytic subunit in the vicinity of the active site. (1) The degree of inactivation is directly proportional to the extents of sulfhydryl alkylation and reagent incorporation. (2) Other protein side chains are not modified, since the number of sulfhydryl groups modified equals the number of reagent molecules linked to the enzyme. (3) The substrates, Fru-1,6-P_2 and dihydroxyacetone phosphate, as well as the competitive inhibitor, P_i, protect the enzyme from inactivation. (4) Autoradiograms of peptide maps of aldolase inactivated by chloroacetol [^{32}P]phosphate reveal one major radioactive peptide.

Haloacetol phosphates (3-halo-1-hydroxy-2-propanone phosphates) were synthesized as potential active-site-specific reagents for enzymes that catalyze reactions involving dihydroxyacetone phosphate (Hartman, 1968a, 1970a). With triose phosphate isomerase, these reagents show absolute specificity for the active site and thereby permitted the identification of a glutamyl γ-carboxylate as a probable catalytically functional group (Hartman, 1968b, 1971; Coulson *et al.*, 1970a,b).

We hoped that haloacetol phosphates would be useful in a comparative study of the active sites of class I and class

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II, Fru-1,6-P_2 aldolases (for a comparison of these two classes of aldolases, see Rutter, 1964), particularly in identifying the presumed nucleophilic group that abstracts a proton from C-3 of dihydroxyacetone phosphate to form the reactive carbanion intermediate (Rose and Rieder, 1955, 1958; Bloom and Topper, 1956; Rutter and Ling, 1958; Christen and Riordan, 1968). Iodoacetol phosphate inactivates rabbit muscle aldolase (the class I prototype), and although inactivation may be a result of modification of essential sulfhydryl groups, extensive oxidation of nonessential sulfhydryl groups precluded an unequivocal interpretation of data (Hartman, 1970b). The corresponding chloro and bromo compounds inactivate aldolase only at pH > 9.5, and the reaction was not previously studied in detail. The recent observation (Lin *et al.*, 1971) that the chloro reagent is selective for an essential sulfhydryl group in yeast aldolase (the class II prototype) prompted us to examine more carefully the reaction of this substrate analog with rabbit muscle aldolase in a continuing effort to elucidate similarities and differences between class I and class II aldolases.

Materials and Methods

Stock solutions of rabbit muscle aldolase at concentrations of 10–20 mg/ml were prepared by dialysis of ammonium sulfate suspensions of the enzyme (from Boehringer Mannheim Corp.) against 0.05 M sodium acetate containing 1 mM EDTA (pH 7.0). Triose phosphate isomerase, α-glycerol phosphate dehydrogenase, glycylglycine, NADH, Fru-1,6-P_2, and dihydroxyacetone phosphate were purchased from the Sigma Chemical Co. Biological grade guanidine hydrochloride was purchased from Schwarz/Mann. Other materials and vendors were TPCK-treated trypsin, Worthington Biochemical Corp.; 5,5′-dithiobis(2-nitrobenzoic acid), Aldrich Chemical Co.; and [^{32}P]$POCl_3$, Ammersham/Searle Corp. Chloroacetol [^{32}P]phosphate (initial specificity activity of 1.6×10^6 cpm/μmole) was synthesized according to the procedure described for the unlabeled compound with the exception of using [^{32}P]$POCl_3$ in the phosphorylation step, which was scaled down 20-fold (Hartman, 1970a).

Protein Assays. Aldolase concentrations were determined from the absorbancy at 280 nm using an $A^{1\%}_{1\,cm}$ value of 9.38 (Donovan, 1964) and assuming the molecular weight to be 160,000 (Kawahara and Tanford, 1966).

Fluorescence Measurements. Fluorescence quenching studies were carried out with the instrument described by Longworth and Battista (1970). The wavelength used for excitation was 280 nm, and the fluorescence was measured at 335 nm. All experimental conditions were identical with those described by Rose and O'Connell (1969).

Aldolase Assays. Aldolase was assayed by the spectrophotometric method of Blostein and Rutter (1963). Each assay solution contained 0.05 M glycylglycine (pH 7.5), 1 mM Fru-1,6-P_2, 0.15 mM NADH, and 28 μg of glycerol phosphate dehydrogenase–triose phosphate isomerase in a total volume of 3.0 ml. The specific activity, at 24°, of several commercial aldolase preparations ranged from 7.1 to 9.6 units per mg. Pure rabbit muscle aldolase has specific activities varying from 14 to 18 units per mg (Penhoet *et al.*, 1969).

Competitive Inhibition Studies. The assay method used was essentially the one described in the preceding paragraph. Rates of NADH oxidation were measured using a Beckman Acta V recording spectrophotometer. Each assay cuvet (3.0 ml) contained 2.6 μg of aldolase, and the Fru-1,6-P_2 concentration was varied from 2.3 to 93.3 μM. Triose phosphate isomerase was eliminated from the assay solution because it is rapidly inactivated by chloroacetol phosphate (Hartman, 1971). Although chloroacetol phosphate is actually a poor substrate for glycerol phosphate dehydrogenase (or a substrate for a contaminant present in commercial preparations of the enzyme), the rate of Fru-1,6-P_2 cleavage was still easily measured. In control experiments, halving the dehydrogenase concentration did not alter the measured rate of NADH oxidation. Thus, any decrease in the effective dehydrogenase concentration brought about by chloroacetol phosphate did not invalidate the assay. Corrections were made for changes in A due to reduction of chloroacetol phosphate. Under the conditions used, 1.27 mM chloroacetol phosphate resulted in a decrease of 0.007 absorbancy unit/min. This rate was always determined just before initiating the cleavage of Fru-1,6-P_2 by the addition of aldolase.

Inactivation of Aldolase by Chloroacetol Phosphate. For a typical experiment in which enzymic activity, sulfhydryl content, and ^{32}P incorporation were to be monitored, a 10-ml solution of aldolase (3.30 mg/ml) was prepared by diluting the stock aldolase (see Materials) with water. The volume was increased to 20 ml by the addition of 10 ml of 0.1 M glycine–1 mM EDTA (pH 10.0), and the pH was then readjusted to 10.0 with 1 N NaOH. To a 15-ml portion of the aldolase solution was added 0.5 ml of 0.03 M chloroacetol phosphate, the pH of which was adjusted to 8.0 with cold, 1 N NaOH just before use. Thus, the final concentrations of aldolase and chloroacetol phosphate in the reaction mixture were 0.01 and 1 mM, respectively. To the remaining 5 ml, which served as the control, was added 0.165 ml of water. Periodically, 2-ml samples of the reaction mixture were diluted into 0.1 ml of 0.5 M ammonium acetate–1 mM EDTA–0.02 M β-mercaptoethanol (pH 4.0) to terminate the reaction. Samples (2 ml) from the control mixture were removed at zero time and after the last sample was taken from the reaction mixture. Aliquots (0.1 ml) of the terminated reaction mixtures were diluted 30-fold with 0.05 M glycylglycine (pH 7.5) and assayed for aldolase activity. The remainder of each sample (2 ml) was dialyzed exhaustively against 0.01 M sodium acetate–1 mM EDTA (pH 7.0) and used for sulfhydryl and radioactivity determinations.

Sulfhydryl Determinations. Free sulfhydryl groups in aldolase were quantitated as described by Ellman (1959). A solution (0.4 ml) containing a known quantity of aldolase was added to 2.1 ml of 8 M guanidine hydrochloride–0.2 M sodium phosphate–3 mM EDTA–0.4 mM 5,5′-dithiobis(2-nitrobenzoic acid) (pH 8.0). The sulfhydryl concentration was calculated from the increase in A at 412 nm, which was completed within 1 min, using an ϵ of 13,600.

Determination of ^{32}P Incorporation. Radioactivity was measured in a Packard liquid scintillation spectrometer. The protein solution (0.1 ml) was dissolved in 1 ml of Beckman BioSolv (BBS-3), and to this mixture was added 10 ml of scintillation fluid composed of 4.6 g of 2,5-diphenyloxazole and 115 mg of 1,4-bis[2-(5-phenyloxazolyl)]benzene in 1 l. of toluene–ethanol (4:3, v/v).

Preparation of Aldolase for Peptide Mapping. Aldolase was inactivated with chloroacetol [^{32}P]phosphate as described except that the final protein concentration was increased to 6 mg/ml. After the enzymic activity had decreased to 10% of the initial value (about 30 min), the excess reagent was decomposed by adding β-mercaptoethanol (final concentration of 0.01 M). The modified aldolase was then treated with sodium borohydride (0.01 M) to reduce the carbonyl group

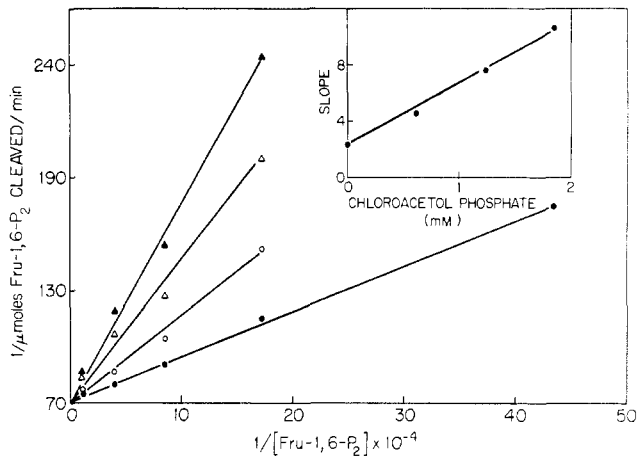


FIGURE 1: Competitive inhibition of aldolase by chloroacetol phosphate at concentrations of 0 (●), 0.61 (○), 1.23 (Δ), and 1.84 mM (▲). Experimental details are given in the Materials and Methods section.

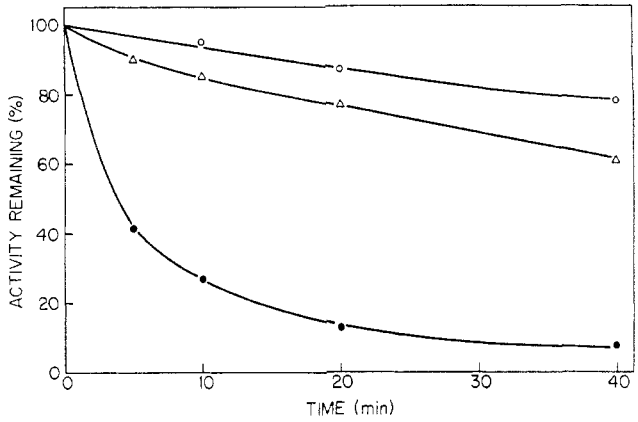


FIGURE 2: Protection of aldolase against trypsin inactivation (●) with 0.2 mM dihydroxyacetone phosphate (○) or 6 mM chloroacetol phosphate (Δ). Experimental details are identical with those used by Rose and O'Connell (1969).

of the incorporated acetol moiety followed by iodoacetate (0.03 M) to carboxymethylate free sulfhydryl groups. The reduction and carboxymethylation were accomplished as described by Hartman (1971) for triose phosphate isomerase modified by chloroacetol phosphate. The protein solution was then dialyzed against 0.1 M ammonium bicarbonate (pH 8.1) and digested with trypsin at a protein : trypsin weight ratio of 100:1 for 2 hr at 40°. The digest was lyophilized to dryness, and to ensure complete removal of ammonium bicarbonate, the dried sample was dissolved in water and relyophilized.

Peptide Mapping and Autoradiography. Peptide mapping was performed with slight modification of the method reported by Katz *et al.* (1959). The sample (1 mg of digest) was applied to a 350 × 400 mm sheet of Whatman No. 3MM chromatography paper and subjected to electrophoresis (1200 V for 1.5 hr) with a Brinkmann Mini-Pherograph apparatus using a buffer (pH 6.5) composed of glacial acetic acid–pyridine–water (10:1:100, v/v). The paper was dried at 70° for 2 hr, and its edge was perforated to allow even solvent flow from the chromatogram. The chromatogram was then subjected to descending chromatography (perpendicular to the direction of electrophoresis) for 12 hr. The solvent was the top layer, obtained by partioning 1-butanol–water–glacial acetic acid (4:5:1, v/v), to which ethanol (5% by volume) was added. Peptides were visualized by dipping the dried chromatogram into 0.5% ninhydrin in acetone and heating at 80°. Autoradiograms of peptide maps were prepared with Kodak No-Screen Medical X-Ray film using a 2-day exposure time.

Results

Interaction of Chloroacetol Phosphate with Aldolase. Chloroacetol phosphate was synthesized with the assumption that such a compound, due to its structural similarity to dihydroxyacetone phosphate, would possess an affinity for the active site of aldolase. To determine the validity of this assumption, the reagent was tested as a competitive inhibitor of the aldolase-catalyzed cleavage of Fru-1,6-P_2. Competitive inhibition was observed with a K_I of 0.6 mM (Figure 1). Under the conditions used for these experiments, chloroacetol phosphate does not react covalently with aldolase, a conclusion based upon the lack of irreversible inactivation and the lack of reagent incorporation (see below).

Binding of chloroacetol phosphate to aldolase is also demonstrated by the reagent's ability to quench aldolase fluorescence and to protect the enzyme against inactivation by trypsin. Consistent with the observation of Rose and O'Connell (1969), dihydroxyacetone phosphate (0.2 mM) affords a high degree of protection against inactivation of aldolase by tryptic digestion. Chloroacetol phosphate (6 mM) gives similar protection (Figure 2). A higher concentration of chloroacetol phosphate was used because of its lower affinity (K_I of 0.6 mM) for aldolase as compared to that of dihydroxyacetone phosphate (K_D of 0.01 mM) (Mehler and Bloom, 1963; Rose and O'Connell, 1969). The protection observed with chloroacetol phosphate is not due to an inhibition of trypsin, because the rate of hydrolysis of *N*-α-benzoyl-L-arginine ethyl ester (0.3 mM) as catalyzed by trypsin (2 μg/ml) is not altered by chloroacetol phosphate (6 mM). This latter experiment was prompted by the report of Adelman *et al.* (1968) that the apparent protection by dihydroxyacetone phosphate of aldolase against carboxypeptidase inactivation was actually due to inhibition of carboxypeptidase.

Dihydroxyacetone phosphate quenches aldolase fluorescence at 335 nm, the maximal extent of quenching being 13% and the concentration required for one-half maximal quenching ($K_{Q/2}$) being 0.016 mM (Rose and O'Connell, 1969). We obtain similar values for dihydroxyacetone phosphate (14% for maximal quenching and 0.008 mM for $K_{Q/2}$) and observe with chloroacetol phosphate a maximal quenching of 6% with a $K_{Q/2}$ of 3 mM. This extent of quenching, although small, appears significant, since it is reproducible and since several bisphosphates that are very good competitive inhibitors of aldolase (*e.g.*, xylitol 1,5-bisphosphate with a K_I of 2.8×10^{-6} M) quench to a similar degree (Suh and Barker, 1971).

Inactivation of Aldolase by Chloroacetol Phosphate. The time course of inactivation of aldolase by chloroacetol phosphate is shown in Figure 3. During the time period illustrated, no loss of activity is observed in control samples incubated with chloroacetol phosphate at pH 7–9.5. At concentrations of 1 mM, both Fru-1,6-P_2 and dihydroxyacetone phosphate are good protective agents; higher concentrations (0.01 M) of P_i, a competitive inhibitor (Mehler, 1963), are required

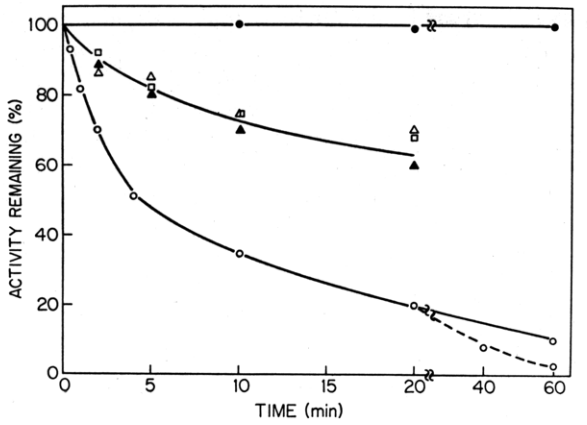


FIGURE 3: Inactivation of aldolase at pH 10 by chloroacetol phosphate (1 mM) in the absence (○) and presence of 1 mM Fru-1,6-P_2 (□), 1 mM dihydroxyacetone phosphate (△), or 10 mM P_i (▲). The dotted line represents an experiment in which additional 1 mM chloroacetol phosphate was added at 20 and 40 min, respectively. Activity in a control solution that lacks the reagent is also shown (●). Experimental details are given in the Materials and Methods section.

for similar degrees of protection, which is consistent with the low affinity of P_i for aldolase.

Correlation of Inactivation, Loss of Sulfhydryl Groups, and Incorporation of Reagent. The inactivation of aldolase by chloroacetol phosphate results from modification of protein sulfhydryl groups, as the extent of ^{32}P incorporation agrees closely with loss of sulfhydryls (Table I). The degree of inactivation is directly proportional to the extents of sulfhydryl modification and ^{32}P incorporation as illustrated in Figure 4.

All of the incorporated radioactivity is accounted for as an *S*-alkyl derivative of cysteine. This identification was made by the procedure used to characterize the product from the modification of yeast aldolase by chloroacetol phosphate (Lin *et al.*, 1971). The inactivated enzyme was treated with [^{3}H]$NaBH_4$, which reduces the carbonyl of the incorporated acetol moiety to a hydroxyl group, thereby introducing a radioactive label into a position that is stable to acid hydrolysis. After hydrolysis of the reduced protein derivative, the

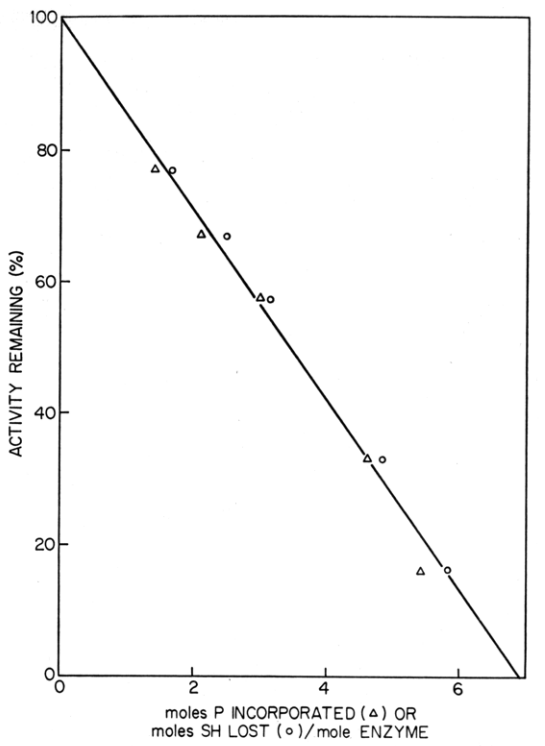


FIGURE 4: Correlation of aldolase inactivation with loss of sulfhydryls (○) and incorporation of chloroacetol phosphate (△). Experimental details are given in the Materials and Methods section.

hydrolysate was chromatographed on a Beckman 120C amino acid analyzer, and fractions were collected for radioactivity measurements. All of the radioactivity emerged coincident with the cysteine derivative prepared by alkylation of glutathione with chloroacetol phosphate followed by borohydride reduction and hydrolysis.

Specificity of the Modification. Autoradiography of peptide maps was used to determine whether the inactivation of aldolase resulted from random or preferential alkylation of sulfhydryl groups. Much of the radioactivity is associated with a single peptide, suggesting substantial selectivity of chloroacetol phosphate for one sulfhydryl group per subunit (Figure 5).

TABLE I: Extent of Reagent Incorporation and SH Modification upon Inactivation of Aldolase by Chloroacetol Phosphate.[a]

Expt	Act. Remaining (%)	Moles of P/Mole of Aldolase	Moles of SH/Mole of Aldolase: Native	Inactivated	No. Modified
1	15	5.4	31.2	25.3	5.9
2	5	7.1	30.8	22.8	8.0
3	20	6.5	31.4	24.2	7.2
4[b]	100	0.1	31.0	31.2	

[a] Aldolase (three different preparations) was inactivated with chloroacetol [^{32}P]phosphate in three separate experiments (conducted at different times over a 2-year period), and the resulting derivatives were assayed as described in the Materials and Methods section. [b] In this experiment, the incubation of aldolase with chloroacetol phosphate was in 0.05 M glycylglycine buffer (pH 7.5) for 2 hr.

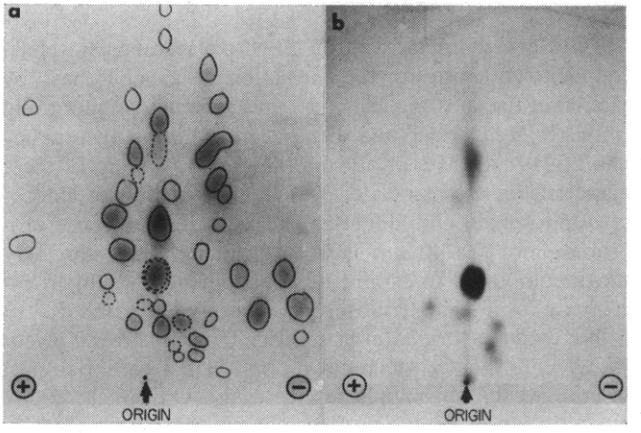
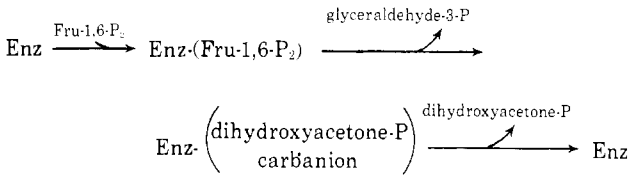


FIGURE 5: Peptide map (a) and autoradiogram (b) of a tryptic digest of aldolase after inactivation with chloroacetol [^{32}P]phosphate. The peptides that were found to be radioactive are circled with broken lines; the major radioactive peptide is indicated by the double line. Experimental details are given in the Materials and Methods section.

Discussion

Our anticipation that chloroacetol phosphate would have an affinity for the active site of aldolase was verified by the observation that the reagent is a competitive inhibitor under conditions where covalent reaction does not occur (Figure 1). The K_I (0.6 mM) compares favorably to the dissociation constants for a variety of compounds bearing a single phosphate group, the values varying from 0.016 mM for dihydroxyacetone phosphate to 6.7 mM for acetol phosphate (Rose and O'Connell, 1969). Kinetic studies have shown an ordered sequence for the aldolase-catalyzed clearage of Fru-1,6-P_2 (Rose



et al., 1965). Such a sequence would appear to preclude interaction of aldehydes with free enzyme; however, Spolter *et al.* (1965) showed that aldehydes are competitive inhibitors of aldolase and therefore can bind to the free enzyme. This apparent contradiction was resolved by Rose and O'Connell (1969) who demonstrated that the enzyme–aldehyde complex cannot react productively with dihydroxyacetone phosphate. Thus, the question arises as to whether chloroacetol phosphate interacts with the dihydroxyacetone phosphate or the glyceraldehyde 3-phosphate binding site.

Rose and O'Connell (1969) suggested that the ability of substrates or competitive inhibitors to quench aldolase fluorescence and to protect aldolase against inactivation by trypsin may be consequences of binding to the dihydroxyacetone phosphate site. Chloroacetol phosphate quenches protein fluorescence, and the concentration required to produce one-half the maximal extent of quenching ($K_{Q/2}$ of 0.3 mM) is in fair agreement with K_I (0.6 mM) determined from competitive inhibition studies. The $K_{Q/2}$ is subject to considerable error due to the maximal quenching being only 6%. At concentrations ten times greater than its K_I, chloroacetol phosphate affords a similar degree of protection against trypsin inactivation as does dihydroxyacetone phosphate at concentrations proportionately higher than its K_D (Figure 2). Thus, if these two criteria are meaningful, chloroacetol phosphate interacts with the dihydroxyacetone phosphate binding site.

Two other characteristics of dihydroxyacetone phosphate binding to aldolase are the formation of a Schiff base by reaction of the substrate carbonyl and a protein amino group (Horecker *et al.*, 1963) and the appearance of an absorbancy peak at 240 nm (Mehler and Bloom, 1963). Neither of these characteristics are associated with the interaction of chloroacetol phosphate and aldolase. The lack of appearance of a chromophore at 240 nm is not surprising, since the only known compound to exhibit this phenomenon is dihydroxyacetone phosphate. The absorbancy is thought to be due to an intermediate formed after the Schiff base because glycoaldehyde phosphate, which does form a Schiff base, does not give rise to the chromophore (Rose and O'Connell, 1969). Our failure to detect a Schiff-base complex between aldolase and chloroacetol phosphate by the criterion of inactivation with borohydride was unexpected in view of the ability of even some aldehydes to form such a complex (Rose and O'Connell, 1969). Possible explanations are that the reagent does not bind at the dihydroxyacetone phosphate site, that a Schiff base is formed but its steady-state concentration is extremely small due to an unfavorable equilibrium, or that the chlorine atom of the reagent, being somewhat larger than the substrate hydroxyl group, causes a distortion in the reagent–enzyme complex which prevents Schiff-base formation.

At pH 10.0, chloroacetol phosphate rapidly and irreversibly inactivates aldolase (Figure 3). The primary question we wished to address was whether inactivation resulted from a preferential modification of the active site. A covalent alteration of an active-site residue is usually exemplified by the following: (1) total loss of enzymic activity; (2) at high molar ratios of reagent to enzyme, inactivation follows pseudo-first-order kinetics; (3) for affinity-labeling reagents in which reversible binding at the active site is an obligatory step preceding covalent reaction, inactivation should be subject to a rate-saturation effect, *i.e.*, at infinite reagent concentration the half-life of inactivation should be finite (Meloche, 1967); (4) substrate protection; (5) 1 mole of reagent incorporated per mole of catalytic subunit; and (6) the same reagent inactivates corresponding enzymes in other species.

Two properties of chloroacetol phosphate have prevented a close examination of the inactivation kinetics. Firstly, the reagent is unstable at pH 10 with a half-life of about 10 min (Hartman, 1970a). Secondly, the initial product of decomposition is dihydroxyacetone phosphate, a compound that protects aldolase against inactivation.

The modification of aldolase probably does not result in total inactivation. A single addition of reagent (1 mM) to aldolase (0.01 mM) causes about 90% inactivation during 1 hr at which time the amount of reagent remaining should be negligible. Stepwise addition of reagent to a final concentration of 3 mM leads to 98–99% inactivation, but we have always been able to detect slight, residual activity.

The ability of dihydroxyacetone phosphate, Fru-1,6-P_2, and the competitive inhibitor, P_i, to protect against inactivation is consistent with modification of a residue near the active site, but, of course, protective effects do not prove catalytic involvement of the residue in question. This is certainly true with aldolase, since substrate-induced conformational changes have been detected (Szabolsci and Biszku, 1961; Adelman *et al.*, 1968).

The inactivation of aldolase by chloroacetol phosphate is clearly due to covalent modification of sulfhydryl groups. There is close agreement between the loss of sulfhydryls and ^{32}P incorporation, thereby eliminating other types of residues from consideration. Furthermore, the only detectable product is an *S*-alkyl derivative on the basis of its chromatographic properties which are identical with those of the cysteine derivative prepared from the alkylation of glutathione with chloroacetol phosphate. It is especially interesting that a direct relationship exists between sulfhydryl modification and inactivation (Figure 4). Therefore, if inactivation is due to conformational changes subsequent to alkylation, the rate of these structural alterations exceeds the rate of alkylation.

The differences in reactivity toward aldolase of the chloro reagent as reported here and the corresponding iodo reagent as reported previously (Hartman, 1970b) obviously reflect inherent differences in the chemical properties of the two compounds. Iodoacetol phosphate, which modifies aldolase primarily *via* oxidation of SH groups to disulfides, also oxidizes sulfhydryls of model compounds (*e.g.*, glutathione) to disulfides, whereas the only demonstrable reactivity of chloroacetol phosphate toward model compounds is alkylation of SH groups (Hartman, 1970a).

Rabbit muscle aldolase is tetrameric (Penhoet *et al.*, 1967; Kawahara and Tanford, 1966). A maximum of only three substrate binding sites have been detected by direct methods (Castellino and Barker, 1966; Ginsberg and Mehler, 1966; Kobashi *et al.*, 1966), but from hybridization studies Penhoet and Rutter (1971) concluded that four binding sites exist. Thus, for a reagent whose reaction is dependent upon initially complexing with the active site, the expected stoichiometry of incorporation is 3–4 moles of reagent/mole of aldolase. The observation that six to eight sulfhydryl groups in aldolase are alkylated by chloroacetol phosphate suggested that about two groups per catalytic subunit are involved in the reaction. However, autoradiograms of tryptic digests of aldolase that was inactivated with [^{32}P]chloroacetol phosphate showed only one major and about seven minor radioactive components. To reconcile this observation with the stoichiometry and the parallelism between sulfhydryl and enzymic activity losses, we conclude that the amount of ^{32}P collectively associated with the minor components is quite close to that of the major component and that the combined rate of random modification of sulfhydryl groups approximates the rate at which a single sulfhydryl per subunit is preferentially modified. The inactivation must result primarily from alkylation of that one group.

Each aldolase subunit contains eight sulfhydryl groups (Lai *et al.*, 1971). Seven peptides, each containing one carboxymethylcysteine residue, have been isolated from tryptic digests of carboxymethylated aldolase and sequenced (Sajgó, 1969). Since the observation by Swenson and Boyer (1957) that aldolase is inhibited by *p*-mercuribenzoate, the role of sulfhydryl groups in enzymic activity has been the subject of many investigations (for a review, see Morse and Horecker, 1968). Perhaps the most exhaustive study concerning this matter is the recent one of Steinman and Richards (1970) in which aldolase sulfhydryl groups were converted to mixed disulfides. Four residues per subunit are unreactive and therefore classified as buried. With respect to their proposed role in catalysis, the four reactive residues were classified as contact, auxiliary, contributing or noncontributing (terms suggested by Koshland, 1960). Of these four residues, only two react in the presence of substrate. One is clearly noncontributing, since its selective modification does not result in either catalytic or structural alterations. The other is considered as contributing to the catalytic process on the basis of slight changes in kinetic parameters and destabilization of protein structure. The investigators concluded that the two residues protected by substrate have an undefined, auxiliary catalytic function. Certainly, these two residues cannot be considered catalytically essential because their conversion to mixed disulfides results in only 50–85% inactivation. Of the two protected residues, one is present in the N-terminal region and the other in the C-terminal region of the polypeptide chain.

Another recent investigation of the relationship between sulfhydryl groups and catalytic activity involves inactivation of aldolase by bromoacetate, another reaction subject to substrate protection (Szajáni *et al.*, 1970). Inactivation correlates with carboxymethylation of the sulfhydryl group located in the C-terminal region, one of the same residues determined by Steinman and Richards (1970) to be protected by substrate. This same group is also involved in the disulfide bridge formed upon air oxidation (Lai *et al.*, 1971). The carboxymethylated derivative possesses slight, but inherent, catalytic activity so that the sulfhydryl group cannot be considered absolutely essential.

On the basis of modification of aldolase sulfhydryl groups in a variety of ways (alkylation, arylation, oxidation, and condensation with glyceraldehyde 3-phosphate), Horecker (1970) has postulated that a sulfhydryl is involved in proton removal from the C-4 hydroxyl group of Fru-1,6-P_2, an obligatory step preceding cleavage to trioses. It has not been unequivocally established that total abolishment of catalytic activity results from any of the sulfhydryl modifications described, including the present one. If Horecker's postulate is correct, proton removal must occur at a slow rate even in the absence of the "essential" sulfhydryl group.

The simplest interpretation of our data is that chloroacetol phosphate reacts preferentially with a sulfhydryl group close to the active site. This residue is probably best described as having an auxiliary function in catalysis. We are engaged in isolating the peptide containing the residue most reactive toward chloroacetol phosphate so as to determine whether it is the same residue which upon carboxymethylation leads to inactivation.

Other interpretations are possible. The reactive sulfhydryl may be far removed from the active site, and inactivation results from conformational changes. Substrate protection would be explained by a substrate-induced conformational change in which a previously exposed group becomes buried. We favor the simpler interpretation for two reasons. The reagent is a substrate analog with a demonstrated affinity for the active site. There is little doubt that this affinity is less at pH 10, where inactivation is observed, than at pH 7.5, where affinity is measured. The K_M of Fru-1-P increases 50-fold upon raising the pH from 7.5 to 10.0 (Mehler, 1963). Secondly, the reagent inactivates yeast aldolase (the prototype of the class II enzymes), and again the inactivation is due to a preferential alkylation of one sulfhydryl per subunit (Lin *et al.*, 1971). This is an intriguing observation, since class I and class II aldolases apparently evolved independently (Rutter, 1964; Jack and Harris, 1971). It is difficult to believe that two nonhomologous enzymes with identical catalytic functions react in closely analogous fashions with a substrate analog unless the reactive side chains have similar, important roles. Speculation as to the nature of this role may be premature.

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pH Dependence of the Oxidation of Iodide by Compound I of Horseradish Peroxidase†

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ABSTRACT: The kinetics and stoichiometry of the oxidation of iodide by horseradish peroxidase compound I have been studied as a function of pH at 25° and ionic strength 0.11. The second-order rate constant for the reaction varied from 2.1×10^6 to 7.7 M^{-1} sec^{-1} over the pH range 2.7–9.9. The pH dependence of the reaction is interpreted in terms of two ground-state ionizations on compound I; one pK_a is 4.6 and the other pK_a value lies outside the pH range of the study. It is established that the reaction of compound I proceeds without the intermediate formation of compound II in agreement with Björkstén, and involves a two-electron transfer from iodide. The possibility of formation of an iodine–peroxidase compound or complex in the time scale of the studies is excluded by the kinetic data.

Steady-state kinetic studies of the catalysis of the oxidation of iodide by hydrogen peroxide have been conducted using chloroperoxidase (Thomas *et al.*, 1970), lactoperoxidase (Morrison, 1968), thyroid peroxidase (Hosoya, 1968; Taurog, 1970), and horseradish peroxidase (Nunez and Pommier, 1968; Björkstén, 1968). The results of the present study of the reaction catalyzed by the latter enzyme (EC 1.11.1.7, donor–H_2O_2 oxidoreductase) were obtained primarily by monitoring the rate of change of concentration of the enzyme species directly.[1] Previous studies in our laboratory, concerned with other substrates, have also been conducted primarily by studying the reactions of the compounds of HRP in isolation

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[1] Abbreviations used are: HRP, horseradish peroxidase; HRP-I and HRP-II, compounds I and II of HRP; PN, the ratio of absorbance at 403 and 280 nm.