Haloacetol Phosphates. Potential Active-Site Reagents for Aldolase, Triose Phosphate Isomerase, and Glycerophosphate Dehydrogenase. II. Inactivation of Aldolase*

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ABSTRACT: Haloacetol phosphates were investigated as active-site specific reagents for rabbit muscle aldolase. Under mild conditions, iodoacetol phosphate (IAP) rapidly inactivates aldolase, but bromo- and chloroacetol phosphate have no effect. Twenty-one of the twenty-nine free sulfhydryl groups in aldolase disappear during its inactivation with IAP. Fructose diphosphate and P_i prevent the inactivation and protect six to eight sulfhydryl groups from modification. Incubation of the inactivated enzyme with cysteine restores 65% of the activity and all but two to three of the sulfhydryl groups. Sulfite or cyanide restores about 50% of the activity and 50% of the sulfhydryl groups restored by cysteine, demonstrating that the major modification is an oxidation of sulfhydryl groups to disulfides. The irreversible losses of enzymic activity and sulfhydryl groups correlate with the covalent incorporation of 2 moles of reagent/mole of aldolase. The lack of incorporation in the presence of substrate implies that the labeled groups are essential. In the presence of a competitive inhibitor (P_i), 12 sulfhydryl groups of aldolase are oxidized with no loss of activity; but, upon removal of the P_i from the solution containing the active, modified aldolase, 65% of the activity is lost. Experiments in which arsenite was used as a reducing agent suggest that, in the presence of P_i, IAP oxidizes two nonessential sulfhydryl groups to sulfenic acids. Inactivation, which occurs upon removal of P_i, is probably the result of essential sulfhydryl groups, which had been protected by P_i, reacting with the sulfenic acids to form disulfides.

Warburg and Christian (1943) and Rutter (1961, 1964, 1965) recognized two classes of FDP[1] aldolases based on differences in physical, chemical, and catalytic properties. Class I aldolases, represented by the enzyme from rabbit muscle, contain essential amino groups that form Schiff bases with the ketone group of the substrate. The Schiff base can be reduced with sodium borohydride to a stable, secondary amine devoid of enzymic activity (Horecker *et al.*, 1963). In addition to labeling and identifying an essential residue, this procedure provides evidence that a Schiff base is an obligatory intermediate in the reaction catalyzed by class I aldolases. Class II aldolases, represented by yeast aldolase, contain a divalent metal ion (Richards and Rutter, 1961a), which is required for catalytic activity (Kobes *et al.*, 1969). These enzymes are unaffected by treatment with borohydride in the presence of substrates and presumably do not form Schiff bases (Rutter, 1964).

The differences in properties of the two classes of aldolase do not necessarily reflect different mechanisms. On the contrary, both rabbit muscle (Rose and Rieder, 1955; Bloom and Topper, 1956; Rutter and Ling, 1958) and yeast aldolase (Rose and Rieder, 1958) catalyze a stereospecific, proton exchange reaction at C-3 of DHAP, suggesting the formation of a carbanion intermediate. Christen and Riordan (1968) and Riordan and Christen (1969) directly demonstrated carbanion intermediates in the aldolase reaction by using tetranitromethane as a trapping agent. Rutter (1964) proposed that the Schiff base of class I aldolases and the metal ion of class II aldolases perform the same functions; that is, they induce formation and stabilization of the DHAP carbanion.

Rabbit muscle and yeast aldolase may be structurally related, for their amino acid compositions and subunit sizes are quite similar (Harris *et al.*, 1969). If the topography of the active sites of rabbit muscle and yeast aldolase are similar, as seems possible from analogies in mechanism and structure, a single reagent might react with the presumed nucleophilic groups that promote carbanion formation and thus permit a direct structural comparison of the active sites of class I and class II aldolase.

Meloche (1967) successfully used bromopyruvate, a reactive derivative of the natural substrate, to label the active site of 2-keto-3-deoxy-6-phosphogluconate aldolase. His investigations prompted me to determine if haloacetol phosphates would react similarly with FDP aldolases. Haloacetol phosphates differ from DHAP only in having a halogen atom instead of a hydroxyl group at C-3 and should therefore possess an affinity for the DHAP binding site of aldolase. If these reagents are bound at the active site with an orientation similar to that of DHAP, the nucleophilic group that promotes carbanion formation may be capable of displacing the halogen atom, thereby resulting in covalent bond formation with the haloacetol phosphate. Studies of the reaction of iodoacetol phosphate with FDP aldolase from rabbit muscle are reported in this paper.

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[1] Abbreviations used in this work are: FDP, fructose 1,6-diphosphate; DHAP, dihydroxyacetone phosphate; F-1-P, fructose 1-phosphate; NADH, reduced niacin-adenine dinucleotide; IAP, iodoacetol phosphate.

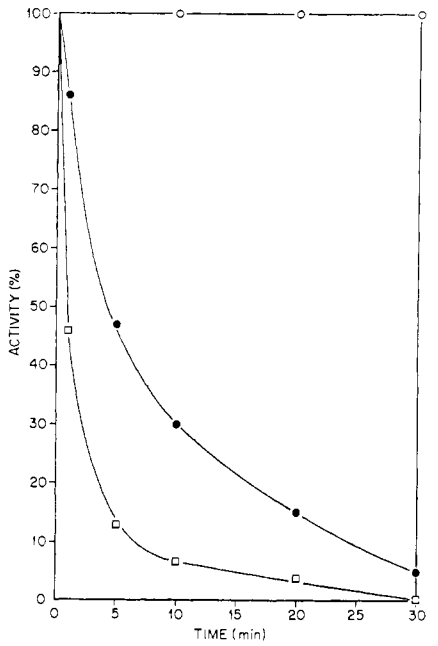


FIGURE 1: Effect of haloacetol phosphates on aldolase activity. The reaction mixtures contained 0.05 mg/ml of aldolase in 0.05 M imidazole buffer (pH 6.5) with (○) no additions, 0.1 mM chloroacetol phosphate, or 0.1 mM bromoacetol phosphate; (●) 0.1 mM IAP; and (□) 1 mM IAP. Other procedural details are given in Materials and Methods.

Materials and Methods

FDP aldolase from rabbit muscle, obtained from Boehringer Mannheim Corp., was dialyzed against the appropriate buffer as needed. The following materials were purchased from Sigma Chemical Co.: NADH, DHAP, FDP, F-1-P, glycylglycine, glycerophosphate dehydrogenase, and triose phosphate isomerase. Other materials and vendors were [^{32}P]$POCl_3$, New England Nuclear; biological grade urea, Schwarz BioResearch; and 5,5′-dithiobis(2-nitrobenzoic acid), Aldrich Chemical Co. Haloacetol phosphates were prepared as previously described (Hartman, 1970). ^{32}P-labeled IAP (initial specific activity 246,000 cpm/μmole) was obtained by use of [^{32}P]$POCl_3$ in the phosphorylation step, which was scaled down tenfold.

All buffers contained 1 mM EDTA unless otherwise indicated.

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

Radioactivity was measured in a Beckman liquid scintillation spectrometer. The protein solution (0.1–0.3 ml) was mixed in 1 ml of Hyamine hydroxide; 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).

Protein samples were prepared for amino acid analysis by hydrolysis with 6 N HCl at 110° for 21 hr in sealed, evacuated (50 μ) tubes. The hydrolysates were concentrated to dryness on a rotary evaporator and analyzed with a Spinco Model 120 C automatic, amino acid analyzer according to the method of Spackman *et al.* (1958).

Aldolase was assayed by the spectrophotometric method of Blostein and Rutter (1963). Each cuvet contained the following components in a total volume of 3.0 ml: 0.05 M glycylglycine (pH 7.5), 1 mM FDP, or 10 mM F-1-P, 0.15 mM NADH, and 30 μg of α-glycerophosphate dehydrogenase with or without 3 μg of triose phosphate isomerase. One unit of activity was defined as the cleavage of 1 μmole of substrate/min. With triose phosphate isomerase in the assay medium, the specific activity, at 24°, of two commercial aldolase preparations were 10.2 and 11.9 units per mg, respectively. Pure rabbit muscle aldolase has specific activities varying from 14 to 18 units per mg (Penhoet *et al.*, 1969).

Sulfhydryl groups were measured by a modification of Ellman's procedure (1959). Into cuvets containing 2.4 ml of aldolase dissolved in 9 M urea, 0.1 M sodium phosphate (pH 8.0), and 5 mM in EDTA, was added 0.1 ml of 0.01 M 5,5′-dithiobis(2-nitrobenzoic acid). The increase in absorbancy at 412 nm was complete within 5 min, and the sulfhydryl concentration was calculated using ϵ 13,600. Twenty separate determinations showed native aldolase to contain 29 ± 1 sulfhydryls per molecule, a value in agreement with those previously published (Benesch *et al.*, 1955; Swenson and Boyer, 1957; Kobashi and Horecker, 1967). Before addition of the sulfhydryl reagent, the aldolase concentration in the 9 M urea solution could be measured from the absorbancy at 280 nm using an $A^{1\%}_{1\,cm}$ value of 8.54. When sulfhydryl determinations were required on aldolase in dilute solutions or in solutions containing IAP, cysteine, cyanide, sulfite, or arsenite, the aldolase was first precipitated and washed with aqueous ammonium sulfate by the procedure to be described below.

The reaction, at 24°, of IAP with aldolase was monitored by measuring changes in enzymic activity, changes in sulfhydryl content, and incorporation of reagent. At protein concentrations of 0.5 mg/ml or less, aliquots containing 5 μg of aldolase were withdrawn and placed directly into cuvets for assaying. Triose phosphate isomerase was omitted from the assay medium in all cases in which IAP was introduced at final concentrations exceeding 5×10^{-6} M because triose phosphate isomerase is inactivated by IAP (Hartman, 1968b). With glycerophosphate dehydrogenase as the only enzyme in the assay medium, the presence of 0.2 mM IAP did not alter the observed aldolase activity.

In experiments in which changes in both activity and sulfhydryl content were monitored, the concentration of aldolase in the modification reaction mixture was 1.6 mg/ml. For activity measurements, 0.1-ml aliquots were quenched by diluting to 3.0 ml with cold, 0.1 M sodium phosphate buffer (pH 7.0). For sulfhydryl determinations, 0.8-ml aliquots were placed in centrifuge tubes containing 9 ml of cold, 50% (g/v) ammonium sulfate buffered with 0.1 M sodium phosphate (pH 2.5). The precipitated protein was collected within 15 min after its removal from the reaction mixture by centrifugation at 4° (12,000g for 3 min). The supernatant was discarded and the precipitate was resuspended, with the aid of a Vortex mixer, in the ammonium sulfate solution (5 ml) and again collected by centrifugation. This process was repeated two additional times. To the washed precipitate was added 3 ml of 9 M urea containing 5 mM EDTA and 0.1 M sodium phosphate (pH 8.0) and the mixture was warmed

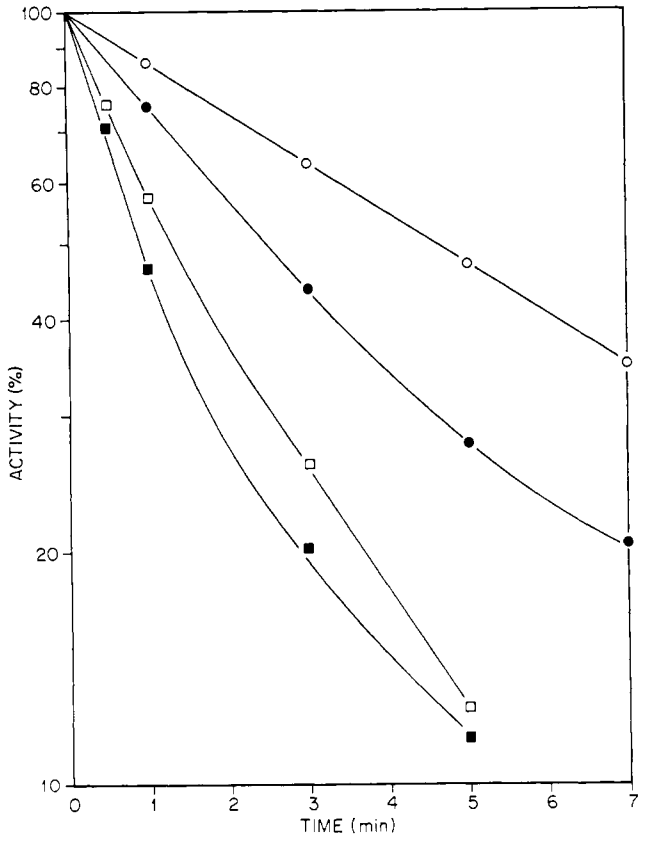


FIGURE 2: Pseudo-first-order plots of the inactivation of aldolase by (○) 0.1 mM, (●) 0.25 mM, (□) 1 mM, and (■) 5 mM IAP. The conditions were as described in the legend of Figure 1.

to 40° to hasten dissolution. After 5 min, traces of insoluble material were removed by centrifugation at room temperature. About 80% of the protein was recovered. Sulfhydryl determinations were performed on 2.4 ml of the supernatant as just described. That this procedure quenched the reaction of IAP with aldolase and resulted in no subsequent change in sulfhydryl content was shown by adding a sample of native aldolase to ammonium sulfate containing 0.1 mM IAP. After centrifugation and washing, the precipitated aldolase still contained 28 sulfhydryl groups.

In the same experiments, the extent to which cysteine could reverse the modification of aldolase by IAP was determined by periodically removing 1.0-ml aliquots and adding cysteine to a final concentration of 0.01 M. Cysteine decomposes IAP almost instantaneously (Hartman, 1970). After incubation with cysteine for 2 hr, the aliquots were assayed for enzymic activity, precipitated, washed, and assayed for sulfhydryl groups.

The rate of incorporation of ^{32}P-labeled IAP into aldolase was determined by measuring the radioactivity in aliquots that had been removed periodically from the reaction mixture, quenched with cysteine, and subjected to exhaustive dialysis.

Results

Kinetics of Inactivation of Aldolase by Haloacetol Phosphates. IAP inactivates aldolase as illustrated in Figure 1.

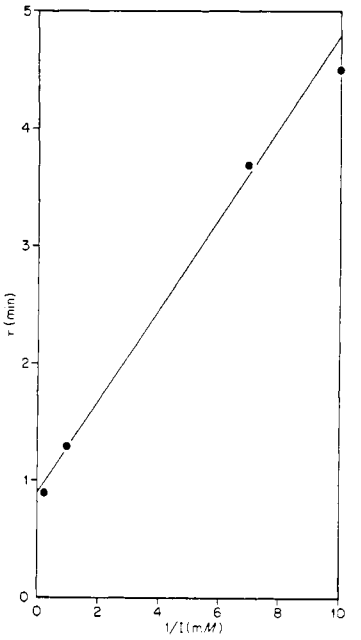


FIGURE 3: Inactivation half-life determined from initial rates in Figure 2 as a function of the reciprocal of IAP concentration.

The loss of activity is the same whether FDP or F-1-P is used as substrate. At pH 10.0, chloro- and bromoacetol phosphate inactivate aldolase, but this reaction has not been thoroughly investigated.

As shown in Figure 2, over a 50-fold range of IAP concentration, the loss of activity is pseudo first order during the first 50–70% inactivation. If the half-life of inactivation (τ) determined from the initial rate of activity loss is plotted as a function of the reciprocal of IAP concentration, a limiting finite value approached by τ is clearly demonstrated (Figure 3). A rate-saturation effect of this type is the result anticipated provided that the reagent and enzyme initially form a reversible complex and the bound reagent then reacts irreversibly with an essential amino acid residue. Inactivation proceeding by this pathway can be expressed by

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\text{---}I \xrightarrow{k_2} E\text{–}I \qquad (1)$$

where E represents free enzyme, I the inhibitor, E- - -I a dissociable complex, and E–I the inactivated enzyme. Since the observed rate of inactivation is proportional to [E- - -I], which has a maximal value equivalent to the total enzyme concentration, it approaches a limiting value as [I] approaches infinity. The rate equation derived by Meloche (1967) is

$$\tau = \frac{1}{[I]}(TK_{inact}) + T \qquad (2)$$

where τ is the observed inactivation half-life; T, the minimum half-life at infinite inhibitor concentration; and K_{inact} is $(k_{-1} + k_2)/k_1$, which represents the inhibitor concentration required to produce the half-maximum rate of inactivation.

Equation 2 shows that a plot of τ *vs.* 1/[I] should yield a

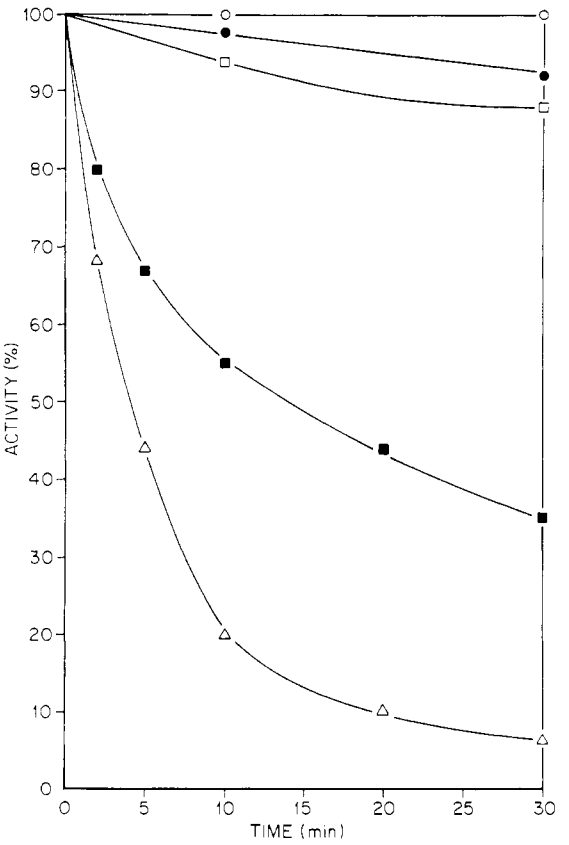


FIGURE 4: Effects of substrates and phosphate on the rate of inactivation of aldolase by IAP. Reaction mixtures contained 0.25 mM IAP and 0.5 mg/ml of aldolase in 0.05 M imidazole (pH 6.5) with one of the following additions: (○) 1 mM FDP or 0.1 M P_i, (●) 1 mM DHAP, (□) 1 mM F-1-P, and (■) 1 mM P_i. (△) No addition.

straight line. Based on data presented in Figure 3, T is 0.9 min corresponding to k_2 of 0.13 sec^{-1}, and K_{inact} for IAP is 0.43 mM, a value having the same order of magnitude as the K_m for DHAP (Richards and Rutter, 1961b).

TABLE I: Inactivation of Active, IAP-Modified Aldolase When Substrate Is Removed from the Reaction Mixture.

Sample	Treatment	Act. (%)
Native	None	100
	Gel filtration in 0.1 M carbonate	100
	Gel filtration in 0.1 M P_i	100
Modified in presence of FDP[a]	None	95
	Gel filtration in 0.1 M carbonate	35
	Gel filtration in 0.1 M P_i	93

[a] Aldolase (1.6 mg/ml) in 0.1 M sodium bicarbonate (pH 8.0), containing 0.01 M FDP was treated with 1 mM IAP for 60 min.

TABLE II: Incorporation of ^{32}P-Labeled IAP into Aldolase.

Sample	Radioactivity (cpm/mg)	Moles of ^{32}P/160,000 g
IAP-modified aldolase[a] (5% active)		
After gel filtration	2100	2.15
After dialysis against 8 M urea–0.01 M cysteine	1950	2.00
IAP-modified aldolase[b] (90% active)	290	0.29
IAP-modified aldolase[c] (88% active)	310	0.31

[a] Aldolase (1.6 mg/ml) in sodium bicarbonate (pH 8.0) was treated with 1 mM IAP for 90 min and passed through Sephadex G-25, equilibrated with the same buffer. [b] Aldolase modified in 0.1 M sodium phosphate (pH 8.0); other conditions as described in footnote *a*. [c] FDP (0.01 M) was included in the reaction mixture; other conditions as described in footnote *a*.

Substrate Protection against Inactivation. The presence of substrates or P_i in the reaction mixture has pronounced effects on the rate of inactivation of aldolase by IAP (Figure 4). At 1 mM concentrations, FDP, F-1-P, or DHAP afford essentially complete protection. Higher concentrations (0.1 M) of P_i, a competitive inhibitor (Mehler, 1963), prevent the inactivation.

Even though aldolase activity is preserved in the presence of substrate or phosphate, its subsequent removal by gel filtration is accompanied by a 60–70% loss of enzymic activity. This inactivation is not attributable to a preferential removal of the substrate as compared with IAP, thus allowing further modifications, for inclusion of P_i in the buffer used for gel filtration prevents the loss of activity (Table I).

Extent of Incorporation of IAP into Aldolase. ^{32}P-Labeled reagent was used to detect any covalent derivatives resulting from the modification of aldolase by IAP. Aldolase that has been 95% inactivated contains 2 moles of ^{32}P/mole of protein, and this incorporation is prevented by inclusion of FDP or P_i in the reaction mixture (Table II). The reagent is apparently attached to the protein through a covalent linkage since radioactivity is not lost by dialysis against 8 M urea containing 10 mM cysteine.

Effects of Cysteine, Cyanide, Sulfite, and Arsenite on IAP-Modified Aldolase. Cysteine partially reverses the inactivation of aldolase by IAP (Figure 5). The effects of cysteine, cyanide, sulfite, and arsenite on the SH content and enzymic activity of modified aldolase are given in Table III. Aldolase, 90% inactivated by IAP contains only 8 sulfhydryl groups/molecule as compared with 29 found in the native enzyme. Of the 21 sulfhydryl groups modified, 18–19 are regenerated by cysteine treatment. If FDP or P_i are included in the reaction mixture, six sulfhydryls are protected against modification by IAP, and subsequent cysteine treatment restores the sulfhydryl content to its original level. About 50% as many sulfhydryl groups were regenerated by treating modified

TABLE III: Effects of Cysteine, Cyanide, Sulfite, and Arsenite on IAP-Modified Aldolase.

Sample	Act. (%) after Treatment with					Moles of SH/Mole of Aldolase after Treatment with				
	None	Cysteine	Cyanide	Sulfite	Arsenite[d]	None	Cysteine	Cyanide	Sulfite	Arsenite[d]
1. Native	100	100	100	100	98	29	29	29	28	29
2. Modified[a]	10	75	42	37		8	26	18	15	
3. Modified in presence of FDP[b]	85	95				13	30			
4. Modified in P_i[c]	100	100				14	30			
5. Sample 4 after gel filtration in 0.1 M Na_2HPO_4, pH 8.0	93				90	15				18
6. Sample 5 after gel filtration in 0.1 M $NaHCO_3$, pH 8.0	35	95	72	61	35	15	29	22	21	14

[a] Aldolase (1.6 mg/ml) in sodium bicarbonate (pH 8.0) was treated with 1 mM IAP for 90 min and passed through Sephadex G-25, equilibrated with the same buffer to remove excess reagent. Samples were made 0.01 M in cysteine, cyanide, or sulfite and after 1 hr were assayed for enzymic activity and sulfhydryl content as described in Materials and Methods. [b] FDP (0.01 M) was included in the reaction mixture and cysteine was added without prior gel filtration. [c] The modification was conducted in 0.1 M sodium phosphate (pH 7.0) and cysteine was added without prior gel filtration. [d] After treatment of aldolase samples with 0.01 M sodium arsenite for 1 hr, they were subjected to gel filtration on Sephadex G-25 equilibrated with 0.1 M sodium bicarbonate before assaying for activity and SH groups.

aldolase with cyanide or sulfite as with cysteine. Also, about 50% of the cysteine-restorable activity was recovered by the action of cyanide or sulfite.

Incubating, with arsenite, aldolase that has been modified in the presence of P_i prevents the loss of activity which normally accompanies the subsequent removal of P_i. Such samples of aldolase contain three more SH groups per molecule than do corresponding aldolase samples that are not incubated with arsenite.

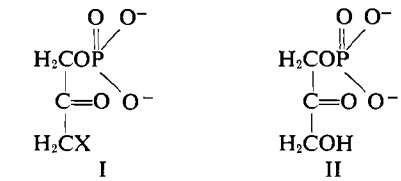
Correlation of Inactivation with SH Loss. The time course of changes in sulfhydryl content and enzymic activity during modification of aldolase by IAP and the reversibility of these changes induced by cysteine are plotted in Figure 6. The rate of inactivation parallels the rate of modification of the slowly reacting SH groups (Figure 7).

The covalent incorporation of IAP into aldolase correlates with the irreversible changes in enzymic activity and sulfhydryl content (Figure 8).

Amino Acid Compositions of IAP-Modified Aldolase. Only insignificant differences were found in comparing the amino acid compositions of native and IAP-modified aldolase (Table IV).

Discussion

I have attempted to apply affinity labeling (Baker, 1967; Singer, 1967) to rabbit muscle aldolase by using haloacetol phosphates (I), compounds that closely resemble DHAP (II). IAP rapidly inactivates aldolase (Figure 1). The rate-saturation effect, observed when the half-time of inactivation is plotted as a function of the reciprocal of IAP concentration (Figure 3), implies that the reagent binds to and subsequently



I II

(X = Cl, Br, or I)

reacts with the active site. Protection by substrate and P_i against inactivation of aldolase (Figure 4) also indicates that IAP modifies essential residues.

The observations that IAP oxidizes free cysteine and glutathione to their disulfides (Hartman, 1970) and that the inactivation of aldolase by IAP is 60–70% reversible by cysteine treatment (Figure 5) prompted a detailed study of the changes in free sulfhydryl content of aldolase during its modification. The extensive loss of sulfhydryl groups (Table III) clearly demonstrates that IAP is not specific for the active site of aldolase; however, FDP or P_i protect six sulfhydryl groups from modification implying their essentiality to either catalysis or the maintenance of native conformation. Most of the sulfhydryl groups with which IAP reacts are oxidized to disulfides. This conclusion is based on the ability of cysteine to restore 18 of the 21 modified sulfhydryl groups and the ability of cyanide or sulfite, reagents that cleave disulfides to form only 1 mole of sulfhydryl/mole of disulfide (Cecil, 1963), to restore one-half as many sulfhydryl groups as cysteine (Table III).

Oxidation of sulfhydryl groups proceeds more rapidly than the inactivation. After 1 min, 50% of the sulfhydryl groups are gone, but the activity is still 75% of the initial level (Figure 6). In the presence of phosphate, of the 14 sulfhydryl

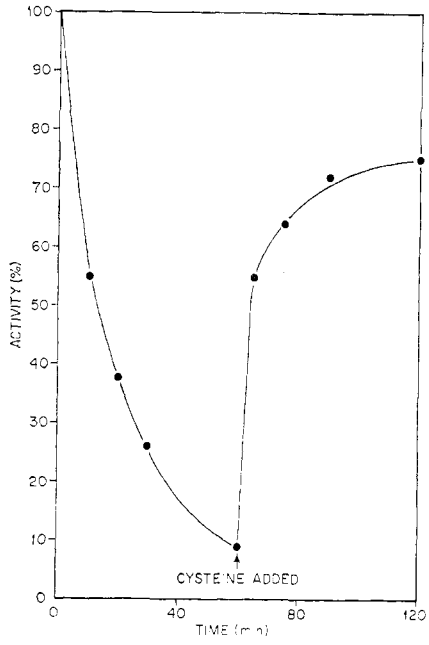


FIGURE 5: Cysteine activation of IAP-inactivated aldolase. The reaction mixture contained 1 mM IAP and 1.6 mg/ml of aldolase in 0.05 M imidazole (pH 6.5). After 60 min cysteine was added to a final concentration of 0.1 M.

groups oxidized during 90 min, 12 were lost after 1 min. If, in the absence of phosphate, as instantaneous oxidation of 12 sulfhydryl groups with no concomitant loss in enzymic activity is assumed, the rate of loss of the other 9 sulfhydryl groups that are ultimately modified approximates the rate of inactivation (Figure 7). These data are consistent with an initial rapid reaction of IAP with certain nonessential sulfhydryl groups of aldolase followed by a slower reaction with essential sulfhydryl groups.

If the differences in sulfhydryl content of aldolase samples modified in the absence and presence of phosphate are compared at various levels of inactivation, the number of sulfhydryl groups protected determined by extrapolation to total inactivation is 6–8, a value in good agreement with that found in experiments recorded in Table III. For example, after 5-min incubation with IAP, aldolase is 56% active and contains 3.3 fewer sulfhydryls than an analogous sample of aldolase modified in the presence of phosphate.

In the experiments in which high concentrations (1.6 mg/ml) of aldolase were modified so that the sulfhydryl content could be readily determined, carbonate buffer (pH 8.0) was used instead of imidazole as in the kinetic experiments. This change was made because, in imidazole buffer with high protein concentrations, turbidity began to appear at 90–95% inactivation and extensive precipitation occurred within 24 hr. Aldolase inactivated in carbonate showed no signs of precipitation even after several days. These differences are probably explained by the fact that during modification conducted in carbonate the sulfhydryl content decreased to a rather constant level of 8 moles/mole of aldolase (Figure 6) but in imidazole continued to decrease to levels of 2–4.

The lack of complete reversibility of sulfhydryl losses resulting from reaction of IAP with aldolase could be attributable to formation of higher oxidation products, such as cysteic acid, or to formation of stable covalent derivatives of sulfhydryl groups. Acid hydrolysates of the modified aldolase contain only traces of cysteic acid, and their total amino acid compositions, except for proline and cystine, are within experimental error of those found in the native protein (Table IV). The cystine content of aldolase samples treated with IAP is greater than native aldolase, as predicted from the results of sulfhydryl assays. The apparent loss of proline in modified aldolase is presumed to be a consequence of cysteine's emergence at the same position as proline (Moore and Stein, 1963).

TABLE IV: Amino Acid Composition IAP-Treated Aldolase.[a]

Amino Acid	Residues/160,000 g: Native Aldolase	IAP-Treated Aldolase[b] (5% Act.)	IAP-Treated Aldolase[c] (95% Act.)
Lys	122	120	120
His	46	46	47
Arg	68	68	67
Cysteic acid	0	0.2	0.2
Asp	116	117	118
Thr	86	85	85
Ser	77	77	76
Glu	169	168	168
Pro	84	78	78
Gly	123	123	123
Ala	171	171	169
Half-Cys	18	24	22
Val	78	80	80
Met	9	9	10
Ile	72	74	74
Leu	140	140	140
Tyr	46	46	46
Phe	29	29	29

[a] All samples were dialyzed against water before hydrolysis. [b] Aldolase was treated with IAP as described in footnote *a* of Table II. [c] Aldolase was treated with IAP in the presence of 0.1 M sodium phosphate (pH 8.0).

Two molar equivalents of [^{32}P]IAP are incorporated into aldolase (Table II) and the rate of incorporation approximates the rates of irreversible losses of enzymic activity and sulfhydryl groups (Figure 8). These data, in addition to the prevention of incorporation by substrate and phosphate, suggest that about two essential sulfhydryl groups of aldolase are covalently modified by IAP and account for the incomplete reversibility of both sulfhydryl losses and inactivation. Extrapolation of the 30% loss in enzymic activity associated with the modification of two sulfhydryl groups to total inactivation, reveals that about six sulfhydryl groups are again implicated as being essential.

Although rabbit muscle aldolase is tetrameric (Penhoet *et al.*, 1967), it contains only three substrate binding sites (Ginsburg and Mehler, 1966; Castellino and Barker, 1966; Kobashi *et al.*, 1966; Ginsburg, 1966). It is, therefore, difficult

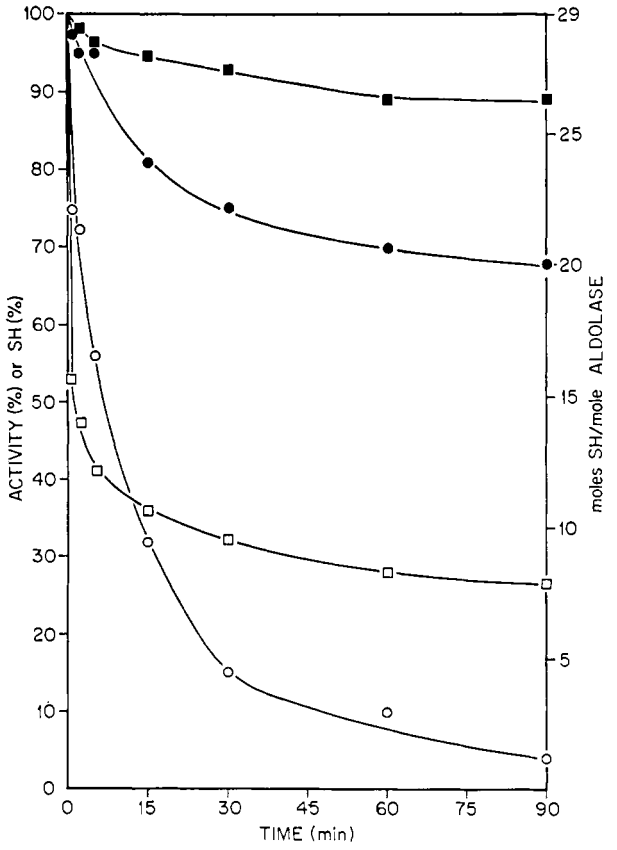


FIGURE 6: Changes in enzymic activity and sulfhydryl content during modification of aldolase by IAP and the reversibility of these changes by cysteine. The reaction mixture contained 1 mM IAP and 1.6 mg/ml of aldolase in 0.05 M bicarbonate (pH 8.0). Periodically samples were withdrawn and assayed as described in Materials and Methods: (○) activity, (●) activity after incubation with cysteine, (□) SH content, and (■) SH content after incubation with cysteine.

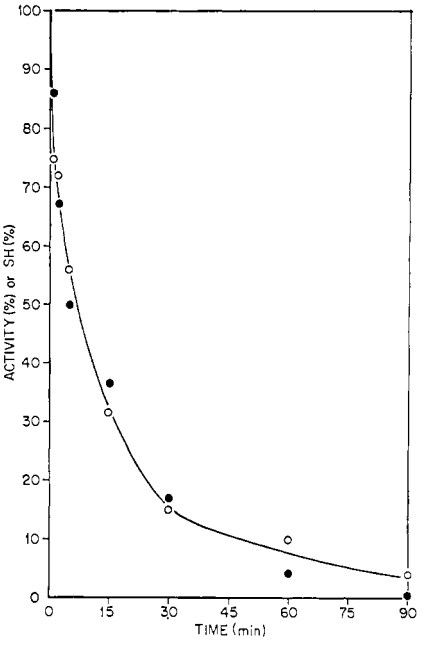


FIGURE 7: Correlation of rate of inactivation (○) with rate of modification (●) of the slowly reacting SH groups. The inactivation data are identical with those presented in Figure 6 and the SH data are derived from Figure 6 by normalizing the values from 57 to 27% (17–8 SH groups remaining) to 100%.

to predict the number of essential residues of any given type. The present designation of six sulfhydryl groups as essential is considered minimal due to the impure aldolase used.

The apparent relation among incomplete restoration of activity, incomplete recovery of sulfhydryl groups, and incorporation of IAP may be fortuitous and the modification of residues other than cysteine may be responsible for the irreversible losses of enzymic activity. Although tryptophan and methionine are potential sites of oxidation, neither of these free amino acids is oxidized by IAP (Hartman, 1970). Tryptophan has not been analyzed, but its modification seems unlikely since native and inactivated aldolase have the same A at 280 nm. Methionine sulfoxide would not have been detected by amino acid analyses of acid hydrolysates. If the incorporation of reagent were caused by an alkylation of lysyl, histidyl, or tyrosyl residues, amino acid analyses would have been inconclusive because a loss of two residues of these amino acids is within experimental error of the determination. An unequivocal assignment of the type of residue(s) that become(s) labeled awaits isolation and characterization of the derivative.

Assuming that the incorporated reagent represents covalent sulfhydryl derivatives, IAP is specific for aldolase sulfhydryl groups, but some groups are oxidized and others covalently modified. That sulfhydryl groups apparently become labeled by IAP indicates an unusual reactivity since IAP merely oxidizes model sulfhydryl compounds. This unusual reactivity of certain aldolase sulfhydryl groups could be a result of their being components of the active site.

Potential explanations of the loss in enzymic activity which occurs upon removal of substrate from active, IAP-modified aldolase (Table I) are the following: (a) Oxidation of nonessential sulfhydryl groups to disulfides introduces a conformational strain into the native, three-dimensional structure, but the presence of substrate at the active-site counteracts this strain. Upon removal of the substrate, the previously introduced strain results in critical conformational changes and subsequent loss of enzymic activity. (b) After removal of substrate, the sulfhydryl groups that had been protected could participate in interchange reactions with the disulfides formed initially. This seems unlikely since inactivation occurs even if the protecting agent is removed at pH 6.0 to lessen the possibility of interchanges. (c) Regardless of the mechanism of sulfhydryl oxidation by IAP, a reactive intermediate must be involved. If a second sulfhydryl group is not sterically situated so as to complete disulfide bond formation, some type of activated sulfhydryl may remain. In the presence of protective agent, if a reactive derivative of a nonessential sulfhydryl group is formed and if this group is adjacent to a substrate-protected sulfhydryl group, disulfide-bond formation and inactivation could occur upon the removal of substrate.

If the loss of activity associated with substrate removal merely reflects a conformational change induced by disul-

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correlated with oxidation of 10 sulfhydryl groups. The major oxidation products were not disulfides but some higher oxidation species.

Depending on the modifying reagent used, 4 to 10 sulfhydryl groups have previously been classified as essential (Kowal *et al.*, 1965; Lai and Hoffee, 1966; Kobashi and Horecker, 1967; Riordan and Christen, 1968). Unfortunately, as in the present study, their role in catalysis cannot be unequivocally distinguished from their role in maintenance of the native conformation.

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