LABELING OF THE ACTIVE SITE OF ALDOLASE WITH GLYCERALDEHYDE 3-PHOSPHATE AND ERYTHROSE 4-PHOSPHATE

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The inactivation of rabbit muscle aldolase by a variety of sulfhydryl reagents and the protection against this inactivation by substrate and substrate analogues provides a useful technique for the detection of functional sulfhydryl groups at the active center (1-3). During the course of these studies we observed a gradual loss of enzyme activity during prolonged incubation with high concentrations of FDP,[1/] with the appearance of a bright yellow addition compound. This effect has also been noted by Woodfin (4), who attributed the inactivation of the enzyme to a reaction involving FDP and more than half the ϵ-amino groups of lysine residues in the protein.

We now wish to report a highly specific inactivation of rabbit muscle aldolase by its substrates, glyceraldehyde 3-phosphate (Ga3P) and erythrose 4-phosphate (E4P). This reaction occurs when stoichiometric quantities of these substances are added to solutions containing the enzyme protein. The loss of activity is irreversible and associated with the incorporation of 4 equivalents of substrate per mole of enzyme,

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[1/] Abbreviations used: FDP, fructose 1,6-diphosphate; Ga3P, glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate; E4P, erythrose 4-phosphate; G6P, glucose 6-phosphate; F1P, fructose 1-phosphate; GPD, glycerophosphate dehydrogenase; TIM, triose phosphate isomerase; TEA, triethanolamine; EDTA, ethylenediamine tetraacetate.

or one per subunit of MW 40,000 (5). The reaction appears to involve the second triose phosphate binding site of the enzyme, and thus provides a new tool for the analysis of the active center and the three-dimensional structure of the protein.

RESULTS

Inactivation of aldolase by FDP: Neutral solutions of rabbit muscle aldolase kept at 30° in the presence of 20 mM FDP showed a slow loss of catalytic activity (Fig. 1). Control solutions stored under identical conditions without FDP were stable for days. Inactivation was accompanied by the appearance of a yellow color which could not be dissociated from the protein by precipitation with ammonium sulfate solution or by gel filtration. It was also associated with the incorporation of organic phosphate groups into the protein, corresponding to approximately 5-6 equivalents per mole of enzyme inactivated (Fig. 1). A similar correlation between loss of activity and incorporation of organic phosphate residues has been ob-

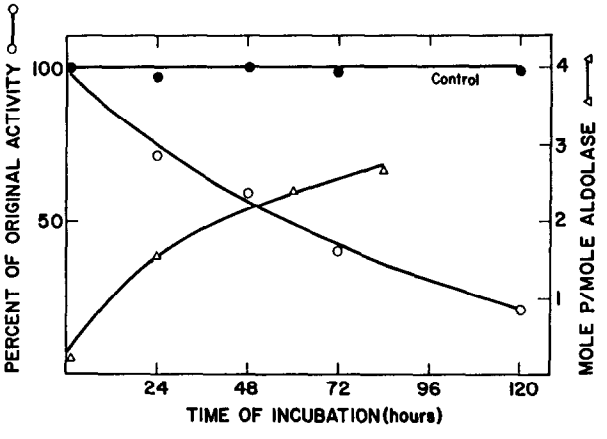

Figure 1. Inactivation of aldolase in the presence of FDP. Aldolase was prepared by the method of Taylor (6) and recrystallized several times to reduce the level of contaminating triose phosphate isomerase. The reaction mixtures contained 7.5 μM aldolase, 20 mM FDP (Boehringer), and 50 mM borate. The pH was 7.5. At the times indicated aliquots were analyzed for catalytic activity with FDP as the substrate, using the method previously described (7). Other aliquots were precipitated with 60% saturated ammonium sulfate solution, dialyzed against distilled water, and analyzed for organic phosphorus (8).

served by Kobashi (9). No gross changes in molecular weight were observed, in contrast to the results reported by Woodfin (4).

The effects of D,L-Ga3P and DHAP: When the FDP cleavage products were incubated with the enzyme under similar conditions, a rapid inactivation was observed with Ga3P, but not with DHAP (Table I). In the presence of a 35-fold molar excess of D,L-Ga3P, the enzyme was inactivated to the extent of 85% in 30 minutes. DHAP protected against the effects of Ga3P; this protection was essentially complete when a 10-fold molar excess was added.

TABLE I

Effect of Incubation with Triose Phosphates on Aldolase Activity [a]

Experiment No.	Aldolase	D,L-Ga3P	DHAP	Specific Activity After 30 Minutes
	mM	mM	mM	units/mg
1	0.018	0	0	12.4
2	0.027	0.96	0	1.9
3	0.027	0.96	0.096	4.7
4	0.027	0.96	0.96	6.3
5	0.027	0.96	9.60	11.2
6	0.027	0	9.60	12.5

[a] The reaction mixtures contained aldolase and triose phosphates, at the concentrations indicated, in 10 mM EDTA and 40 mM TEA buffer, pH 7.5. Aliquots were tested for catalytic activity after incubation at 30° for 30 minutes. Aldolase was a recrystallized sample purified by chromatography at pH 8.0 on DEAE-cellulose with a salt gradient. The specific activity was 12.4 μmoles of FDP cleaved per min per mg of protein. The triose phosphate isomerase activity of this preparation was 0.0025 μmoles of Ga3P converted per min per mg of protein.

Inactivation by stoichiometric quantities of Ga3P: The effects of Ga3P were observed even at concentrations equivalent to those of the enzyme (Fig. 2). When the ratio of D-Ga3P to enzyme was 5:1, which repre-

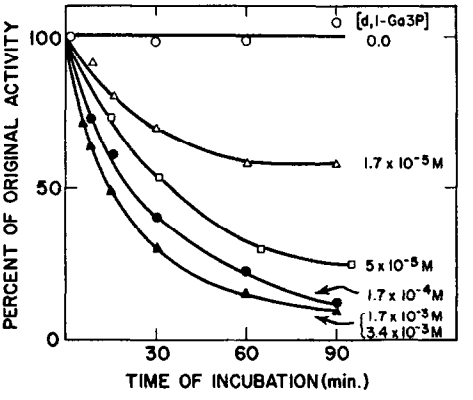

Figure 2. Effect of Ga3P concentration on the rate of inactivation of aldolase. The reaction mixtures contained 0.01 mM aldolase and 10 mM EDTA in 40 mM TEA buffer, pH 7.5. The temperature was 25°. D,L-Glyceraldehyde 3-phosphate was added as indicated; the concentrations given are those of the D- isomer. The specific activity of the aldolase was 12.8 units per mg and the level of TIM contamination was 0.0077 units per mg. D,L-Glyceraldehyde 3-phosphate diethylacetal was purchased from the Boehringer-Mannheim Co.

sents slightly more than one equivalent per subunit, loss of activity reached 75% in 90 minutes. In the presence of 1.7 equivalents per mole of enzyme (0.4 per subunit), 40% of the original activity was lost. However, analysis of the solution at 90 minutes indicated that only 30% of the D-Ga3P added had been consumed, suggesting that the L- isomer was also reacting, and experiments were therefore carried out with the separated D- and L- isomers.

In these experiments aldolase preparations containing very low levels of triose phosphate isomerase activity were employed to minimize the formation of DHAP from D-Ga3P (Fig. 3). L-Ga3P was more active than D-Ga3P, and with 4.4 equivalents of this substance added per mole of enzyme loss of activity reached 95% in 2 hours. In the presence of an equimolar quantity of DHAP, the enzyme was protected almost completely against inactivation by D-Ga3P, and the rate of inactivation by the L- isomer was markedly reduced. Incomplete inactivation in the presence of the slight excess of D-Ga3P may be attributed to the formation of DHAP during incu-

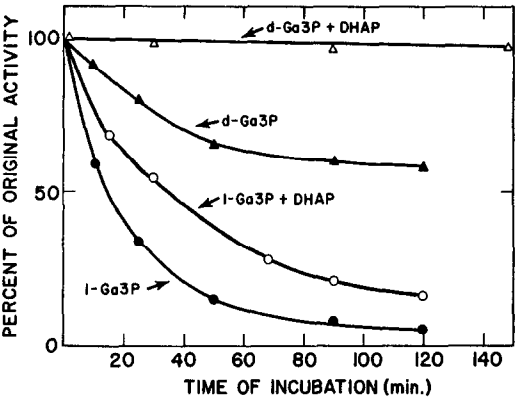

Figure 3. Inactivation of aldolase with D- or L-Ga3P and the effect of DHAP. The experimental conditions were as in Fig. 2, with the following concentrations: Aldolase, 0.025 mM; D-Ga3P, 0.12 mM; L-Ga3P, 0.11 mM; DHAP, 0.11 mM. The specific activities of aldolase and TIM in the crystalline enzyme preparation were 12.3 units/mg and 0.00043 units per mg, respectively. D-Ga3P was prepared from FDP with muscle aldolase using acetaldehyde to trap the DHAP moiety. The product was purified by chromatography on Dowex 1-formate. L-Ga3P was prepared from the commercial D,L- mixture by converting the D- isomer to α-glycerophosphate with α-glycerophosphate dehydrogenase, TIM, and a slight excess of DPNH. DPN and DPNH were removed with charcoal and L-Ga3P was purified as the hydrazone according to the method of Hall (10). DHAP was synthesized according to Ballou (11). D-Ga3P was analyzed with α-glycerophosphate dehydrogenase, TIM, and DPNH, and L-Ga3P was analyzed with transaldolase and F6P, measuring the formation of D-Ga3P spectrophotometrically.

bation; the conversion of D-Ga3P to DHAP was shown to be complete in 2 hours in this experiment.

Inactivation by E4P and the number of combining sites in the enzyme molecule: E4P, a natural substrate for aldolase (12), was found to be as effective as L-Ga3P in the inactivation reaction (Fig. 4). This substrate provided two important advantages: 1) it is not a substrate for isomerase, and 2) it is readily prepared in radioactive form from ^{14}C-glucose 6-phosphate (13). With a 16-fold excess of E4P, inactivation was almost complete in 2 hours. Under these conditions 3.4 equivalents of radioactivity per mole of enzyme were incorporated (Table II). In each experiment the number of equivalents incorporated was nearly one per subunit inactivated. The results are consistent with the presence of 3-4 sites associated with loss of enzyme activity.

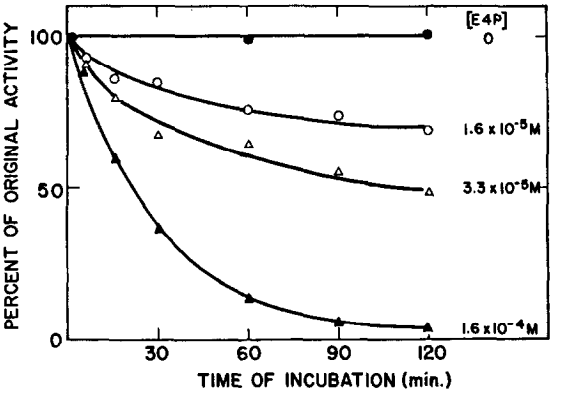

Figure 4. Inactivation of aldolase with D-erythrose 4-phosphate (E4P). The reaction conditions were as in Fig. 2. The concentration of aldolase was 0.01 mM. E4P was added as indicated. E4P was prepared by the procedure of Seiben et al. (13) and analyzed with transaldolase (14).

TABLE II

Incorporation of E4P into Aldolase[a/]

E4P Added	Inactivation	Radioactivity Incorporated	E4P Incorporated
mM	%	cpm/mg protein	equiv./mole
0	0	0	0
0.016	32.5	240	1.1
0.033	52.3	440	2.0
0.16	97.0	750	3.4

[a/] After two hours the enzyme protein in each experiment in Fig. 4 was precipitated with two volumes of cold saturated $(NH_4)_2SO_4$ solution. The precipitate was dissolved in water and dialyzed overnight against 0.05 M NH_4HCO_3 buffer. Aliquots were taken for measurement of radioactivity and for spectrophotometric determination of protein. The specific activity of E4P was 35,600 cpm per μmole.

Specificity of the reaction: No inactivation was observed when the enzyme was incubated with a 20-fold molar excess of acetaldehyde, erythrose, or glucose 6-phosphate. On the other hand, D-glyceraldehyde and

ribose 5-phosphate at 2 mM caused about 20% inactivation in 2 hours. Both of these are substrates for aldolase. Hexitol diphosphate did not inactivate the enzyme, although at 1 mM concentration it protected against inactivation by D,L-Ga3P.

DISCUSSION

Unlike the reaction with FDP, reported by Woodfin (4) and observed in our laboratory, inactivation with Ga3P or E4P was not accompanied by the appearance of color. No change in molecular weight was detected. The specificity of the inactivation reaction described here and the fact that it can be demonstrated with concentrations of substrate equivalent to those of the enzyme, suggest that modification of residues at the active site is involved. This is supported by the protective effects of DHAP and the substrate analogue, hexitol diphosphate. Unlike the inactivation observed when the Schiff base intermediate is reduced (15), inactivation by Ga3P or E4P does not require the addition of borohydride. The nature of the covalent bonds formed and the sites of attachment are currently under investigation.

Acknowledgements

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