DIFFERENTIAL REACTIVITY OF THE SULFHYDRYL GROUPS OF YEAST ALDOLASE TOWARD p-HYDROXYMERCURIBENZOATE*

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SUMMARY - Reaction of p-hydroxymercuribenzoate with sulfhydryl groups in yeast aldolase was studied by the stopped-flow method. Two classes of SH groups can be distinguished. Fast reactive SH groups (approximately 3) have the rate constant around 4×10^5 $M^{-1}sec^{-1}$, which is at least 10^3 faster than the slow reactive SH groups (approximately 2 in holoenzyme). Apoaldolase has 2 additional slow reactive SH groups. Fructose diphosphate blocks the reaction of at least one slow reactive SH group both in holo- and apo-enzymes. These results suggest that the SH groups of yeast aldolase are involved in the fructose diphosphate binding site and possibly involved as a ligand on the crucial Zn atom.

There are two general types of fructose diphosphate aldolases in nature (1, 2). The major difference between these two classes is that the class I aldolases involve a Schiff base formation between dihydroxyacetone phosphate and a lysyl residue on the protein molecule while the class II enzymes require a divalent metal ion for catalytic activity. Another important difference between these two classes of enzymes is that most of the class II aldolases require the presence of sulfhydryl-containing compounds for maximum stability and catalytic activity while the class I enzymes do not, however, sulfhydryl groups within the protein might

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serve a role in catalysis (3). According to this classification, yeast aldolase is definitely a class II enzyme in that it contains approximately 2 g-atoms Zn per mole of enzyme, which are necessary for catalysis (4, 5). Furthermore, yeast aldolase activity has been known to be dependent upon the maintenance of unaltered sulfhydryl residues (3).

In the study of the sulfhydryl groups of yeast aldolase, Ingram has suggested that there are two distinct classes of sulfhydryl residues. Approximately four residues react very rapidly with PMB† while four others react only in the presence of 6M urea or a metal chelator (6). Recently we have shown using the affinity labeling technique that haloacetol phosphates inactivate yeast aldolase by alkylation of one SH group in the active site region of the enzyme catalytic subunit (7). In order to elucidate the role of the sulfhydryl groups in the yeast aldolase molecule in greater detail, we have investigated the differential reactivity of the sulfhydryl groups toward PMB by using the technique of stopped-flow kinetics.

MATERIALS AND METHODS

Homogeneous yeast aldolase was prepared according to the procedure of Rutter et al. (8) with the modification described by Kobes et al. (4). The enzymic activities of these preparations, as assayed according to Richards and Rutter (9), were 50-70 μmoles of FDP cleaved per min per mg of protein. All enzyme solutions were dialyzed against 0.05 M phosphate buffer (pH = 7.0) before use. The molecular weight of yeast aldolase was assumed to be 80,000 and the ϵ at 280 nm was taken to

† Abbreviations used: PMB, p-hydroxymercuribenzoate, FDP, fructose-1,6-diphosphate.

be 1.02×10^4 $M^{-1} cm^{-1}$ (10). PMB and FDP were obtained from Sigma Chemical Co. The concentration of PMB was determined by using ϵ = 16,900 $M^{-1}cm^{-1}$ at 232 nm (11).

Apoaldolase was prepared as described by Kobes et al. (4) except metal free 0.05 M phosphate buffer (pH = 7.0) was used. Apoaldolase prepared in this fashion has 14-18% enzymatic activity of the Zn reconstituted enzyme. FDP solution was made metal free by extracting with dithizone in CCl_4. Metal free PMB was made by dissolving PMB in metal free solution.

Reaction of sulfhydryl groups with PMB was performed at pH = 7.0 as described by Boyer (11). Reactions were followed at 250 nm by the use of a Durrum-Gibson stopped-flow apparatus at $10^{\circ}C$.

RESULTS AND DISCUSSION

Figure 1 shows the oscilloscope traces of the reaction of PMB with the SH groups in the aldolase. The number of SH groups were calculated by using $7.74 \times 10^3 M^{-1}cm^{-1}$ as the molar extinction coefficient. This value was calculated from a titration curve and is

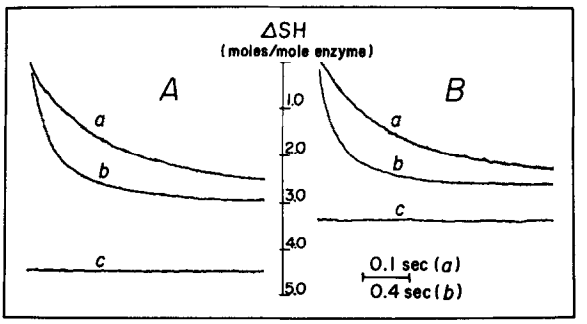

Figure 1. Stopped-flow traces of the reaction of PMB with yeast aldolase at 250 nm in the absence (A) and presence (B) of FDP. Traces (a) and (b) are in different time scales. Trace (c) shows the level of end point of the reaction. Concentration of yeast aldolase: 1.65×10^{-6} M; PMB: 1.59×10^{-5} M; FDP (when added): 5mM.

consistent with that reported by Boyer (11). At least two kinds of SH groups with different reactivities toward PMB can be distinguished in the presence (Fig. 1B) or absence (Fig. 1A) of substrate. The number of the fast-reactive SH groups n and the reaction rate constant k were calculated as follows. By assuming that the fast-reactive SH groups are homogeneous with respect to the reactivity toward PMB, a second order rate equation is applied to the reaction,

$$k = \frac{2.303}{t(a-b)} \log \frac{b(a-x)}{a(b-x)} \qquad (1)$$

where a stands for the total PMB concentration, b for the total fast-reactive SH concentration, which is equal to n times aldolase concentration and x for the concentration of reacted SH (or PMB) at time t after the initiation of the reaction. By rearranging the equation, we get,

$$\log \frac{(b-x)}{(a-x)} = \log \frac{b}{a} - \frac{k(a-b)}{2.303} t \qquad (2)$$

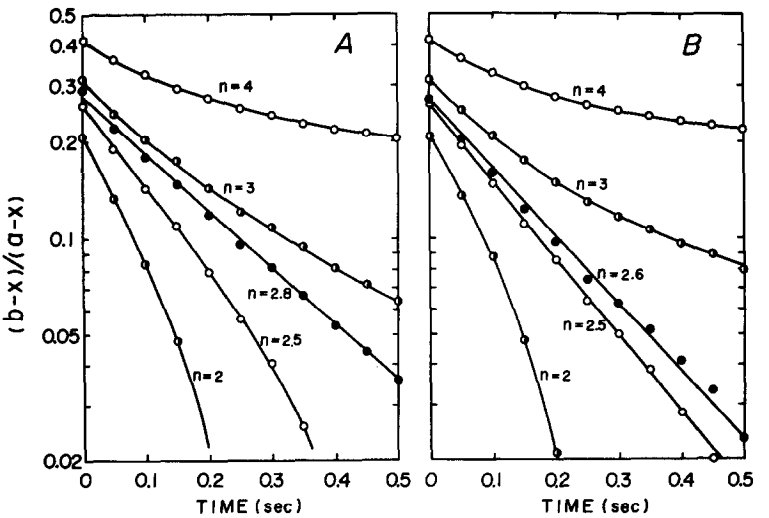

Figure 2. The plot of log $\frac{b-x}{a-x}$ versus t in the absence (A) and presence (B) of FDP. The data were taken from Fig. 1.

The plot of log $\frac{b-x}{a-x}$ versus $\underline{t}$ must give a straight line, if the reaction follows a second order reaction with respect to SH and PMB concentration.

By using the data from Figure 1, the value of $\underline{n}$ was determined by best-fit to be 2.8 and 2.5 with and without substrate, FDP, respectively (Fig. 2). Similar experiments and calculations were performed with PMB and apoaldolase in the presence and absence of FDP. The composite results with Zn aldolase and metal free enzyme are listed in Table I. The number of slow reactive SH groups were calculated by subtracting the number of fast reacting SH groups from the total number of reactive SH groups.

TABLE I

Reactivity of the SH groups of yeast aldolase toward PMB

	Holoenzyme		Apoenzyme	
	-FDP	+FDP	-FDP	+FDP
Total SH (moles/mole enzyme)	4.5	3.3	6.3	5.2
Fast-reactive SH (moles/mole enzyme)	2.8	2.6	2.5	2.5
Rate constant ($M^{-1}sec^{-1}$)	3.8×10^5	4.2×10^5	3.8×10^5	4.4×10^5
Slowly reactive SH (moles/mole enzyme)	1.7	0.7	3.8	2.7
Rate constant ($M^{-1}sec^{-1}$)	130	50	10	23

Number of total SH groups were determined independently by Boyer's Procedure (11).

As shown in Table I, apoaldolase has approximately two additional slow reactive SH groups both with and without substrate as compared with the holoenzyme. With both types of enzymes the presence of FDP blocks the reaction of at least one slow reactive SH group. These studies suggest that the sulfhydryl groups of yeast aldolase are involved in the FDP binding site and possibly involved as a ligand on the crucial Zn atom. Fast reactive SH groups, probably those exposed on the surface of the enzyme molecule, are involved in neither substrate nor Zn binding.

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