THE COMBINING SITES OF YEAST FRUCTOSE DIPHOSPHATE ALDOLASE

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Yeast fructose diphosphate aldolase is competitively inhibited by hexitol diphosphate. Equilibrium dialysis studies indicate that two moles of HDP are bound per 80,000 gm molecular weight of the enzyme. The intrinsic dissociation constant for HDP is 1.9×10^{-4}M as compared to the kinetically determined K_I, 6.8×10^{-4}M, and the Km for FDP, 3.2×10^{-4}M. Dialysis in the presence of the metal chelator, EDTA, affects neither the intrinsic dissociation constant, 1.4×10^{-4}M, nor the number of combining sites, two, for HDP. The results obtained support the contention that the divalent metal ion of aldolase contributes little to the binding process but is principally involved in the catalytic mechanism.

INTRODUCTION

Fructose -1,6-diphosphate (FDP)[1] aldolase (Fructose -1,6-diphosphate D-glyceraldehyde-3-phosphate lyase E.C.4.1.2.13) from yeast is the prototype enzyme for class II aldolases according to the nomenclature of Rutter (1). The enzyme contains a divalent metal ion (Zn^{2+}) and the rate of the catalytic reaction is enhanced by the addition of a monovalent ion (optimally potassium). Recent studies by Harris *et al* (2) suggest that the enzyme has a molecular weight of 80,000 and is probably composed of two sub-units. Apoaldolase, i.e. Zn^{2+} free enzyme, has been prepared (3) and the preparation thus

[1] The following abbreviations are used: FDP, D-fructose-1, 6-diphosphate; EDTA, ethylene diaminetetraacetic acid, disodium salt; NADH, reduced nicotinamide-adenine dinucleotide; HDP, hexitol diphosphate (a mixture of mannitol and sorbitol diphosphates).
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obtained is catalytically inactive. Varying degrees of catalytic activity may be recovered following the addition of Zn^{2+}, Co^{2+}, Ni^{2+}, Mn^{2+} or Fe^{2+} to the apoenzyme. In all cases the resulting holoenzyme is activated by potassium ion.

Initial attempts to quantitate the number of zinc atoms per mole of enzyme indicated an approximate 1:1 ratio (4,5). Kobes *et al.* (3) in a recent study suggest that more than one mole, i.e. 1.2 - 1.6 of zinc is usually found per 80,000 gm molecular weight. These results imply that zinc is lost as a result of the crystallization of the enzyme from concentrated ammonium sulfate solutions, a conclusion reached in the case of other metalloenzymes (6). However, recent experiments employing electron paramagnetic resonance techniques, with Mn^{2+} aldolase suggest the presence of two moles of metal per mole of enzyme (2). In addition to the aforementioned discrepancies no studies are available at present to confirm or refute the speculation that the divalent metal ion functions as an electrophile thereby aiding the enolization of the carbonyl of the substrate FDP (1). The present preliminary study was undertaken to study the role of Zn^{2+} in substrate binding and to determine the number of substrate combining sites for yeast FDP aldolase. In this connection hexitol diphosphate, presently an unknown inhibitor of yeast aldolase, but a potent inhibitor of rabbit muscle aldolase (7), was used to circumvent the problem of substrate cleavage and subsequent removal of dihydroxyacetone phosphate by contaminating traces of triose phosphate isomerase (8).

MATERIALS AND METHODS

Yeast FDP aldolase was prepared and assayed as previously described (9). Uniformly labeled FDP-^{14}C was obtained from New England Nuclear and had a specific activity of 177 μCu per μmole. Hexitol diphosphate and hexitol diphosphate-^{14}C were prepared and purified

as described previously (8). The concentration of hexitol diphosphate was determined as total organic phosphate by the method of Fiske and Subbarow following substrate oxidation (10). Scintillators and Hyamine hydroxide were obtained from the Packard Instrument Co. The scintillation system of Bray (11) was used in all counting procedures. All other reagents were the best grades available from commercial sources.

Equilibrium dialysis experiments were performed at 6°C for 20 hr in cylinderical lucite cells similar to those described by Myer and Schellman (12). The cells were custom made by Mr. G. Harland of this Department. The dialysis membranes, the dialyses and counting procedures were performed as described by Changeux, Gerhart and Schachman (13). All dialysis binding calculations have been described previously (13).

RESULTS

The effect of hexitol diphosphate upon the aldolase catalyzed cleavage of FDP is shown in Fig. 1. The results obtained were plotted according to the method of Dixon (14). A K_I of 6.8×10^{-4}M for HDP was determined. This value compares favorably with the Km of FDP of 3.2×10^{-4}M (1).

Since the kinetic experiments suggested that HDP was a potent competitive inhibitor of yeast aldolase, its ability to bind to the aldolase was tested in the equilibrium dialysis system. A Scatchard plot of data obtained in a typical binding experiment is illustrated in Fig. 2. The intrinsic dissociation constant, calculated from the slope, was determined from the average of several experiments to be 2.0×10^{-4}M. This value is in reasonable agreement with the kinetically determined inhibitor constant referred to previously, i.e. 6.8×10^{-4}M.

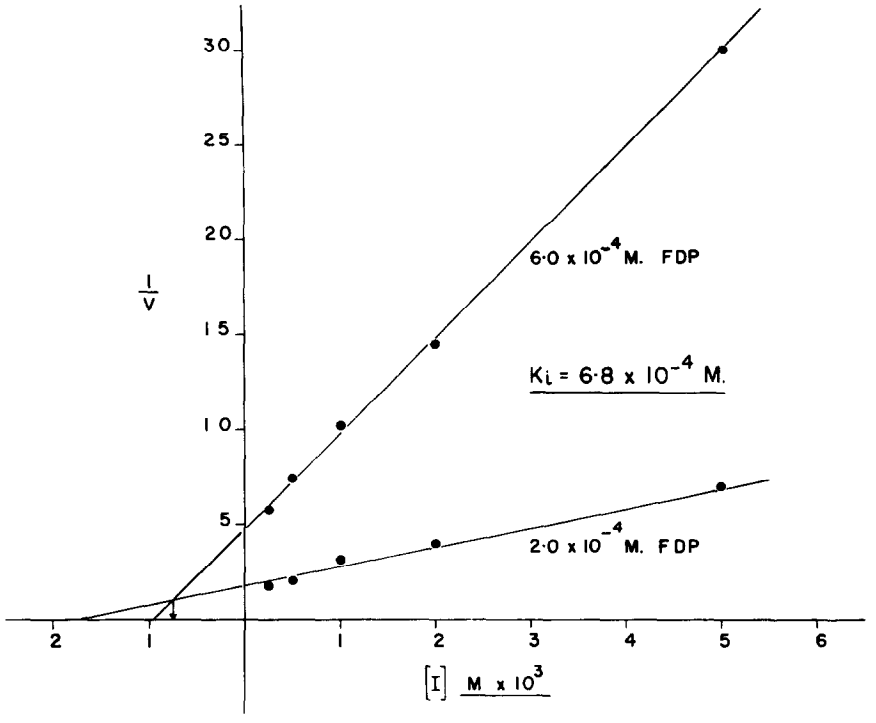

FIG. 1. The effect of hexitol diphosphate on the yeast aldolase catalyzed cleavage of fructose-1, 6-diphosphate. Initial reaction velocities were calculated as μmoles of FDP cleaved per min in reaction cuvettes containing: FDP and HDP as indicated; 0.063 M potassium acetate; 1.25 x 10^{-3}M Cleland's reagent (dithiothreitol); 3.0 x 10^{-2}M phosphate buffer, pH 7.5; 3.0 x 10^{-4}M NADH; 5 μg of a mixture of α-glycerophosphate dehydrogenase and triosephosphate isomerase; and aldolase such that the maximum optical density change was not in excess of 0.80 per minute.

Previous studies indicated that the catalytic activity of yeast FDP aldolase is severely inhibited by metal chelators such as EDTA (15) and that such inhibition is at least partially competitive with respect to the substrate FDP (15). The results presented as a Scatchard plot in Fig. 3 show that EDTA has no deleterious effect upon the binding of HDP, and presumably of FDP, to yeast aldolase. In addition, the number of binding sites is identical to that obtained with untreated aldolase. It appears, however, that at high concentrations of HDP, non-specific binding does occur between the enzyme and HDP in the presence of EDTA. No explanation for this phenomenon is apparent at the present

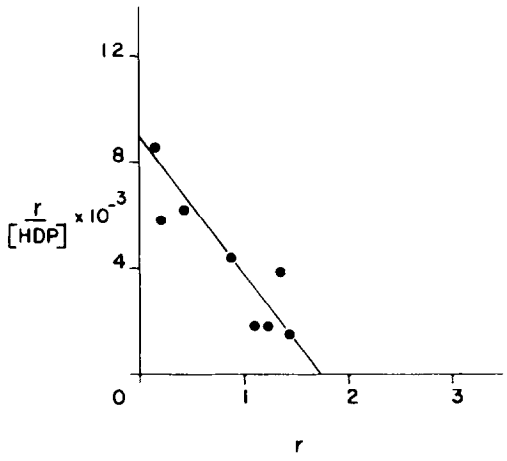

FIG. 2. Binding of hexitol diphosphate by yeast FDP aldolase. The dialysis cells contained 0.063 M potassium acetate; 1.25 x 10^{-3}M Cleland's reagent; 3.8 x 10^{-2}M phosphate buffer, pH 7.5; 3.36 x 10^{-5}M aldolase (based upon a molecular weight of 80,000 (2)) in one chamber; varying concentrations of unlabeled HDP as indicated, and approximately 0.05 μCu of labeled HDP to the chambers not containing aldolase. Dialysis was allowed to proceed for 20 hr at 6°C. Samples (0.05 ml) were withdrawn from each side and counted as described under Materials and Methods.

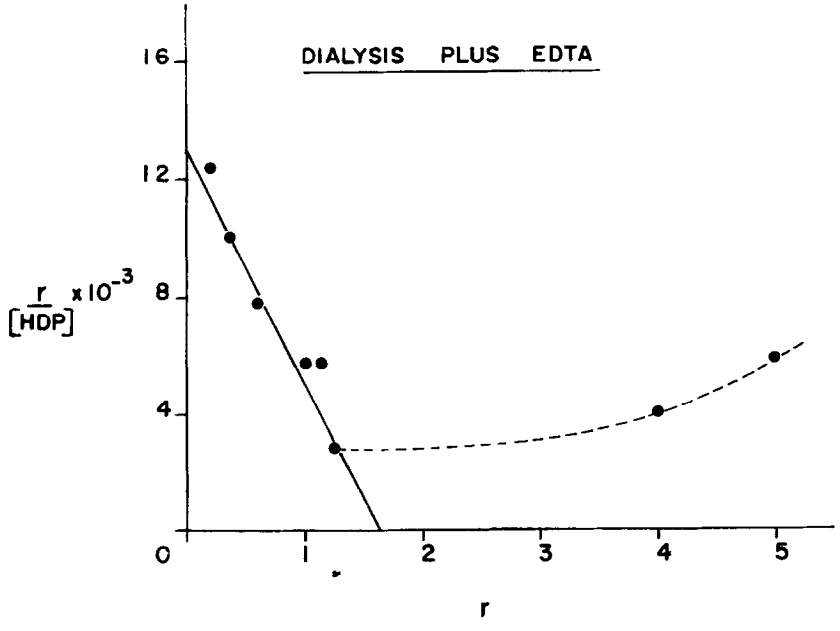

FIG. 3. Binding of hexitol diphosphate by yeast FDP aldolase in the presence of EDTA. The equilibrium dialysis cells were prepared as described in the legend to Fig. 2 except that 1.25 x 10^{-2}M EDTA was included in each cell. The concentration of aldolase was 2.38 x 10^{-5}M and the dialysis time at 6°C was 20 hr.

time. The intrinsic dissociation constant of HDP, expressed as an average from several experiments, was similar to that obtained with untreated aldolase, i.e. 1.4×10^{-4}M.

DISCUSSION

The product of sodium borohydride reduction of fructose -1, 6-diphosphate, hexitol diphosphate, is shown by the results obtained in the present study to be a strong competitive inhibitor of yeast FDP aldolase. Equilibrium dialysis experiments strongly suggest the presence of approximately two binding sites for HDP per 80,000 gm molecular weight, and thence presumably the same number for the natural substrate, FDP. The intrinsic dissociation constant for HDP, 1.9×10^{-4}M, compares favorably with the kinetically determined Km for FDP of 3.2×10^{-4}M.

The determination of approximately two binding sites for HDP, and presumably FDP, per molecular weight of yeast aldolase is a strong indication that one mole of substrate is bound per sub-unit (2) and that probably one zinc atom is required per substrate molecule, perhaps as an electrophile as suggested by Rutter (1). Another interesting observation arising from the present study is the fact that HDP although devoid of the carbonyl function binds as well to yeast aldolase as does the natural substrate FDP; that is if K_I and Km are assumed to be indicators of binding efficiency. If this is the case, then the function of zinc, as mediated through the carbonyl group (1), must be restricted to the catalytic reaction mechanism rather than being involved in the binding process.

The formation of an EDTA-Zn-enzyme complex apparently has no effect upon the availability of the binding sites since FDP is able to compete favorably with EDTA (15) and EDTA, at the concentration used here, neither decreases the apparent number of binding sites nor affects

significantly the intrinsic dissociation constant for HDP. The preceding discussions strongly suggest that any participation of Zn^{2+} in the binding process is most probably of a minor secondary nature. Similar conclusions regarding the lack of or secondary participation of the Schiff base forming ε-lysyl amino group in binding have also been reached with Class I aldolases (7, 16).

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