Interaction between Rabbit Muscle Aldolase and Dihydroxyacetone Phosphate

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(Received for publication, April 27, 1962)

It is generally accepted that the mechanism of enzyme activity includes a combination of enzyme and substrate. This concept forms the basis for the conventional kinetic analyses of enzymatic reactions (1). Direct evidence for the existence of enzyme-substrate combinations is as yet meager. The binding of pyridine nucleotide coenzymes as substrates to various dehydrogenases has been shown to result in changes in absorption and fluorescence (2). In several other cases, the enzyme has been demonstrated to be a participant in the overall reaction, accepting, for example, an acyl group in one step of the reactions of chymotrypsin (3) and triose phosphate dehydrogenase (4), or undergoing oxidation in the reactions of peroxidase (5). Equilibrium dialysis and ultracentrifugation (6) also have been used to measure the binding of enzyme and substrate; such techniques have shown specific binding of substrate, as well as nonspecific absorption.

Rabbit muscle aldolase is a well known representative of a group of enzymes that catalyze aldol reactions with various substrates (7). Similar reactions catalyzed by the transferring enzymes, transaldolase (8) and transketolase (9, 10), recently have been shown to involve the intermediate formation of a derivative of the enzyme with the fragment to be transferred. Particularly in the case of transaldolase, the overall reaction bears a great similarity to the reaction of aldolase, but it remains to be determined whether the two processes involve similar mechanisms.

In a previous report, it was shown that the combination of dihydroxyacetone phosphate and aldolase absorbs ultraviolet light, and it was suggested that the absorbing material is the catalytically active enzyme-substrate complex (11). The measurements reported in this paper indicate that enzymatically altered, active aldolase (12) produces spectral changes with dihydroxyacetone phosphate similar to those caused by native aldolase, whereas inactivated enzyme produces no change. The increases in optical density measured as a function of concentration have been used to calculate the dissociation constant of aldolase and dihydroxyacetone phosphate in the absence of a second substrate.

EXPERIMENTAL PROCEDURE

Aldolase was crystallized from rabbit muscle by the method of Taylor, Green, and Cori (13) or was purchased from Boehringer and Soehne. Dihydroxyacetone phosphate was purchased from California Corporation for Biochemical Research as the cyclohexylamine salt of the dimethyl ketal, and was converted to the free ketone by the method of Ballou and Fischer (14). Carboxypeptidase was kindly furnished by Dr. J. Gladner and Dr. J. E. Folk as a crystalline suspension; the washed crystals were dissolved in a cold solution of 10% LiCl to which was added approximately 5% of 1% NaHCO₃. This solution was used to alter aldolase as described by Drechsler, Boyer, and Kowalsky (19).

Spectrophotometric measurements were made with a Cary model 14 recording spectrophotometer, by use of cuvettes with a 10-mm light path. Some of the difference spectra were recorded with an expanded scale, in which full scale (10 inches) is equivalent to an absorbancy of 0.1.

Dihydroxyacetone phosphate was assayed spectrophotometrically with α-glycero-phosphate dehydrogenase and DPNH (15). It was reported previously that the increase of optical density is a function of both the concentration of aldolase and the concentration of dihydroxyacetone phosphate (11). Fig. 1A includes a family of absorption spectra obtained by adding successive increments of substrate in small volumes to a solution of aldolase. These curves are difference spectra, read against an equal volume of the aldolase solution to which water was added in place of substrate. The spectra are displaced vertically to separate the curves. In Fig. 1B the increase in optical density at 245 mµ relative to that at 270 mµ is plotted as a function of dihydroxyacetone phosphate concentration. The selection of 245 mµ was made to permit measurements with concentrations of aldolase as high as 4 mg per ml; lower wave lengths cannot be used with high concentrations of protein because of the effect of fluorescence described previously (16). In Fig. 2, A and B, similar results with carboxypeptidase-treated aldolase are shown.

The requirement for active aldolase to produce the observed spectral change was tested with three types of inactivated enzyme, produced by treatment with urea, sodium dodecyl sulfate, and N-bromosuccinimide. The urea experiment was carried out in 5 M urea, which is slightly more than sufficient to cause complete inactivation of aldolase in kinetic assays. Inactivation with sodium dodecyl sulfate was only partial in 2 × 10⁻⁴ M detergent, but was complete at 10⁻³ M. Since the protein concentration in the direct spectrophotometric assay is much higher than in the kinetic assay, the detergent was used at a concentration of 2 × 10⁻³ M. The kinetic assay used in the presence of detergent was the coupling with hydrazine, essentially as described by Jagannathan, Singh, and Damodaren (17). N-Bro-
Aldolase-Dihydroxyacetone Phosphate Complex

Fig. 1. Absorption curves of 2 mg of aldolase per ml in 0.02 M Tris-HCl buffer, pH 7.7, recorded by a Cary model 14 spectrophotometer versus a reference cuvette containing an aliquot of the same solution. The amounts of dihydroxyacetone phosphate indicated (in micromoles) were added to the experimental cuvette in 10-µl aliquots of a stock solution; a comparable volume of water was added to the reference cuvette. The total volume was 1 ml. Each space represents an absorbancy of 0.01, recorded with the conventional scale. All measurements were made at 25°. B. The differences between the optical density at 245 m\(\mu\) and 270 m\(\mu\) measured for each curve in Part A are plotted as a function of the molar concentration of dihydroxyacetone phosphate.

Kinetic assays of aldolase have shown that salts inhibit the enzyme as a function of ionic strength (19). The inhibition is competitive with respect to substrate. The effect of ionic strength on the binding of dihydroxyacetone phosphate to aldolase is also seen in the direct spectrophotometric assay. In 0.1 M potassium phosphate, pH 7.0, 4 \(\times\) 10\(^{-4}\) M dihydroxyacetone phosphate added to 1.3 \(\times\) 10\(^{-3}\) M aldolase caused an increase in optical density at 245 m\(\mu\) of 0.002, in contrast to the increase of 0.02 found in the absence of inorganic phosphate. Increasing the dihydroxyacetone phosphate 10-fold caused an increase in optical density to 0.013, whereas this concentration of substrate in solutions of low ionic strength saturates the enzyme and gives an optical density change of 0.045. Magnesium sulfate at a concentration of 0.1 M is even more effective at inhibiting both the overall activity in the kinetic assay and the formation of the enzyme-substrate complex.

The increase in optical density produced by the combination of aldolase and dihydroxyacetone phosphate was originally detected as a property of the hypothetical enolate, which had been proposed to explain the isotope exchange data (20). The enolate of dihydroxyacetone phosphate itself is not known, and the interpretation depends upon analogy with other carbonyl compounds. There is a pronounced spectral shift when solutions of dihydroxyacetone phosphate are made alkaline, but the change in absorption is not reversible and is accompanied by loss of capacity to serve as substrate for glycerophosphate dehydrogenase. Presumably the change in absorption is a measure of the elimination of the phosphate group to form methylglyoxal.

**DISCUSSION**

Several characteristics of the ultraviolet absorption seen on adding dihydroxyacetone phosphate to aldolase support the interpretation that this absorption is a property of an enzyme-substrate complex that is a component of the catalytic process. As reported previously (11), this absorption is specific, in that dihydroxyacetone phosphate does not show a change in spectrum when added to other proteins, and other substrates do not alter their spectra when added to aldolase. The ability of aldolase to alter the spectrum of its substrate is seen to depend on the enzyme retaining its catalytic properties, since inactivation by any of three reagents (that do not cause the enzyme to precipitate) eliminates the effective interaction with the substrate. The dependence of the spectrum on the presence of free dihydroxyacetone phosphate was shown earlier by elimination of the spectrum through addition of an aldehyde capable of condensing enzymatically with the dihydroxyacetone phosphate. The present evidence shows that the change in optical density increases with substrate concentration until a maximal value is reached.

An estimate of the dissociation constant of the aldolase-dihydroxyacetone phosphate complex can be made from the data of Fig. 1. In the usual analyses of enzyme-substrate binding, it is assumed that the enzyme removes negligible amounts of substrate, so that the concentration of free substrate is equal to the amount added per unit volume. In the present case, the concentrations of enzyme and substrate are similar, and significant fractions of the substrate are bound in the concentration range that causes changes in absorption at 245 m\(\mu\). Therefore, an alternative method was used to calculate the dissociation constant, based on the assumption that the maximal optical density change observed in the presence of high concentrations of dihydroxyacetone phosphate is the absorbancy of the complex, corresponding in concentration to total enzyme. The concentration of complex produced by lower substrate concentrations is then taken as proportional to the absorption measured with each concentration. For any given initial substrate concentration, the dissociation constant can be calculated from the equation,

\[
K = \frac{[\text{substrate}_{\text{initial}} - \text{complex}] [\text{enzyme}_{\text{total}} - \text{complex}]}{[\text{complex}]}
\]

For a number of enzyme concentrations ranging from 0.5 to
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4 mg per ml, values of $K$ of $1 \times 10^{-3}$ mm were obtained. Some uncertainty is introduced by the subjective errors in selecting the proper base line for each curve and by lack of precise knowledge of the number of binding sites per enzyme molecule. The calculations were made on the basis of one molar equivalent binding site per 150,000 g of highest specific activity aldolase. Within the limits set by the uncertainties, no difference could be ascertained between the dissociation constants of native and carboxypeptidase-altered aldolase.

It is of interest to note that the spectrum of the aldolase-dihydroxyacetone phosphate complex is not obtained when hexose diphosphate is used as a substrate, and that the absorption characteristic of dihydroxyacetone phosphate and the enzyme disappears on the addition of a reactive aldehyde. It must be concluded that the lifetime of this complex is short compared with the period during which the enzyme exists in other states during the reaction sequence of interconverting the condensed and cleaved substrates. The effect of salts in decreasing the absorption of the enzyme-substrate complex is to be noted here as supporting the contention that the binding of substrate is primarily electrostatic, although the formation of the enzyme-substrate complex described in this paper necessarily involves additional interaction, with stereospecific release of a proton (20, 21).

**SUMMARY**

The absorption in the region of 240 nm seen when dihydroxyacetone phosphate is added to high concentrations of rabbit muscle aldolase (0.3 to $2.5 \times 10^{-4}$ M) has been studied with native and carboxypeptidase-degraded enzyme. The effects of inactivation of the enzyme by denaturation of the protein or by addition of competing salts support the proposal that the absorption is characteristic of an enzyme-substrate complex. A dissociation constant for this complex of $1 \times 10^{-5}$ M was calculated.

**Acknowledgment** We acknowledge with appreciation the technical assistance of Miss Celia Asnien.

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