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FRUCTOSE-BISPHOSPHATE ALDOLASE FROM RABBIT MUSCLE

A THERMODYNAMIC STUDY ON THE FORMATION OF THE ENZYME–DIHYDROXYACETONE PHOSPHATE COMPLEX

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SUMMARY

The influence of pH, ionic strength and temperature on the formation of the aldolase–dihydroxyacetone phosphate complex has been investigated.

It has been shown that the formation of the complex is controlled by groups with a pK of 7.0 which may belong either to the substrate or to the enzyme. The pH dependence of the binding of the substrate is more complex above pH 7.0, and is not interpretable in terms of a few pK values.

It has been confirmed that the increase of the ionic strength increases the dissociation of the complex. This shows that the interaction of the charged groups of the substrate and of the enzyme is a relevant feature in the formation of the complex. It has also been confirmed that the active site is positively charged in the entire pH range investigated.

Both a decrease in the enthalpy and an increase in the entropy contributes to the formation of the complex; the entropy change, however, provides the major contribution.

INTRODUCTION

In this paper we present a study of the effect of the environmental conditions (pH, ionic strength, temperature) on the association constant of the reaction of formation of the aldolase–dihydroxyacetone phosphate complexes. The aim of this work is to provide additional information for the understanding of the mechanism of action of aldolase (D-fructose-1,6-biphosphate D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13). Evidence for the involvement, in the catalytic activity, of particular groups of the protein have been generally obtained through modification with semispecific reagents [1]. Alteration of the catalytic activity of the enzyme under these conditions does not necessarily indicate that the groups modified are directly involved in the catalysis but could also be due to more trivial causes such as steric hindrance or gross changes in the conformation of the protein.

Our experimental approach deals exclusively with the native enzyme and the

natural substrate, and provides the necessary basic data for further detailed kinetic studies on the interconversion of the different intermediate compounds formed by the reaction of the enzyme with dihydroxyacetone phosphate (carbinolamine, protonated and non-protonated Schiff base, intermediate carbanion).

Our measurements show that, between pH 6.0 and 9.7, the formation of the aldolase–dihydroxyacetone phosphate complex is controlled not only by the dissociation of groups with pK around 7.0, but also by additional groups with pK around pH 8.0 and 9.5, pK values which are not clearly resolved with the present technique. They suggest also that the active site of the enzyme is positively charged and that the electrostatic interaction with the negatively charged substrate plays an important role in the formation of the complex. Furthermore they show that both a decrease in the enthalphy and an increase in the entropy contribute to the formation of the complex; the entropy change providing the major contribution.

EXPERIMENTAL

Materials

Fructose bisphosphate aldolase was prepared from rabbit muscle by the procedure of Taylor et al. [2] and was recrystallized 5 times. The specific activity of the preparations used in these experiments was 12 units per mg of protein. A unit was defined as the amount which catalyzes the cleavage of 1 μmole of fructose 1,6-bisphosphate per min under standard assay conditions. Dihydroxy-[^{14}C]acetone phosphate was prepared from commercial [^{14}C]fructose 1,6-bisphosphate following the procedure of Horecker et al. [3] as modified by Ginsburg and Mehler [4].

Methods

Fructose bisphosphate aldolase activity was measured in the test system described by Racker [5]. Radioactivity determinations were made in a Packard Tri-Carb liquid-scintillation counter in 10 ml of Bray's solution [6].

The protein concentration was measured from the absorbance at 280 nm considering that the absorbance of 1 mg of pure enzyme/ml (light path 1 cm) is 0.91 [7]. Sephadex G-50 filtration was performed at 22 °C on columns (1.2 cm $\times$ 37 cm) equilibrated with dihydroxy-[^{14}C]acetone phosphate at the pH and ionic strength indicated in the single experiments. The flow rate was 4 ml/min. Fractions of 1 ml were collected.

Each fraction was analyzed for protein (280-nm absorbance) and for dihydroxyacetone phosphate (radioactivity). Bound dihydroxyacetone phosphate was calculated from the difference in radioactivity between the fractions with and without aldolase. The number of molecules of substrate bound per molecule of enzyme was estimated from the specific activity of the radioactive substrate on the basis of a molecular weight of the enzyme of 159 000 [8].

The aldolase preparation used in these experiments contained about 0.001 % triose-phosphate isomerase. Glyceraldehyde 3-phosphate or fructose 1,6-bisphosphate were never detected in the effluent of the Sephadex G-50 columns, when the assay was performed immediately after the run.

Dihydroxyacetone phosphate was found to be stable, at pH 9.7, for the duration of the experiment.

The ionic strength was related to the dissociation constant of the aldolase di-

hydroxyacetone phosphate complex by making use of the Debye–Huckel equation [9, 10] in its limiting form:

$$-\log K'_{\text{dissoc}} = -\log K_{\text{dissoc}} + 1.02\, Z_e Z_s \sqrt{w}$$

where K'_{dissoc} and K_{dissoc} were, respectively, the apparent and the true dissociation constants of the enzyme–substrate complex; Z_e and Z_s were, respectively, the charges of the enzyme and of the substrate; and w, was the ionic strength.

The pH of the samples was calculated from the potential between the Beckman 41263 glass electrode and the 39070 Ag–AgCl reference electrode as measured with a Beckman Expandomatic pH meter. Calculations were made assuming linearity between pH and the electromotive force E, according to the equation:

$$\frac{\text{pH(X)} - \text{pH(S}_1\text{)}}{\text{pH(S}_2\text{)} - \text{pH(S}_1\text{)}} = \frac{E_X - E_1}{E_2 - E_1}$$

where X is the sample, S_1 and S_2 are the standards and E_X, E_1, E_2 are the electromotive forces of the cells containing the above solutions.

Standards, prepared from Beckman reagents, were Beckman buffers at pH 4.01, 6.86 and 10.00 at 25 °C.

Measurements at different temperatures were made after equilibration (by means of thermostatic equipment) of the electrodes and of both the sample and standard solutions to the desired temperatures.

The correction for Na^+ error was not applied since the highest pH in our experiments was 9.7.

RESULTS

Equilibrium constants of the formation reaction of the aldolase–dihydroxyacetone phosphate complex

The reaction of formation of the aldolase–dihydroxyacetone phosphate complex, as a function of pH and ionic strength, was studied by gel filtration on Sephadex G-50 columns equilibrated with the radioactive substrate (Table I).

The apparent dissociation constants (K'_{dissoc}) were calculated, for each experiment, on the basis of 4 binding sites per molecule of enzyme [11] making use of the equation:

$$\frac{(4 - \bar{\nu})\,[S]}{\bar{\nu}} = K'_{\text{dissoc}}$$

Where $\bar{\nu}$ is the number of molecules of substrate bound per molecule of enzyme; and [S] is the concentration of dihydroxyacetone phosphate. The pH explored was between 6.0 and 9.7 and the ionic strength was between 0.005 and 0.42.

The values of $-\log K'_{\text{dissoc}}$, obtained at the same pH but at different ionic strengths, were plotted against $\sqrt{w}$. Straight lines were obtained, thus showing that the limiting form of the Debye–Huckel equation was followed (Fig. 1).

By extrapolation to zero ionic strength of the plot of $-\log K'_{\text{dissoc}}$ against $\sqrt{w}$,

TABLE I

EFFECT OF THE IONIC STRENGTH ON THE DISSOCIATION CONSTANT OF THE ALDOLASE–DIHYDROXYACETONE PHOSPHATE COMPLEX

The incubation mixture (1 ml) contained aldolase (spec. act. 12 units per mg of protein) as indicated in the table and either 0.02 M Tris–HCl buffer (pH 6.0; 6.5; 7.5; 8.0; 8.5; 9.0) or 0.02 M triethanolamine–HCl buffer (pH 7.0); or 0.02 M glycine–NaOH buffer (pH 9.35; 9.5; 9.7). The ionic strength was calculated on the basis of a pK of 8.08 for Tris; of 7.8 for triethanolamine and of 9.6 for glycine and was adjusted to the values reported in the table by the addition of NaCl. The mixtures were submitted to gel filtration on Sephadex G-50 columns (1.2 cm × 37 cm), equilibrated with the buffer systems indicated above, plus the radioactive dihydroxyacetone phosphate (spec. act. 270 cpm/nmole) at the concentration indicated in the table. The temperature was 22 °C. The columns were operated as described under Experimental. The fractions collected were analyzed for radioactivity and for protein content.

pH	Ionic strength	Dihydroxy[^{14}C]-acetone phosphate equil. concn* (10^{-5} M)	Aldolase concn** (μM)	Aldolase–dihydroxyacetone phosphate complex		$\bar{\nu}$***
				cpm	nmoles	
6.0	0.0198	0.5	12.5	10 100	37.5	3
6.0	0.22	0.5	12.5	2 370	8.8	0.7
6.0	0.42	0.5	12.5	810	3.0	0.24
6.5	0.0195	1.0	12.5	12 630	46.5	3.7
6.5	0.1195	1.0	12.5	6 750	25.0	2.0
6.5	0.2195	1.0	12.5	5 020	18.6	1.49
6.5	0.4195	1.0	12.5	2 420	9.1	0.73
7.0	0.017	1.0	12.5	11 910	43.7	3.50
7.0	0.067	1.0	12.5	7 500	27.8	2.32
7.0	0.117	1.0	12.5	7 380	27.3	2.27
7.0	0.417	1.0	12.5	1 860	6.9	0.55
7.5	0.016	1.0	12.5	9 720	36.0	2.84
7.5	0.116	1.0	12.5	5 680	21.0	1.64
7.5	0.216	1.0	12.5	3 430	12.8	1.02
7.5	0.416	1.0	12.5	1 160	4.3	0.34
8.0	0.0108	1.0	12.5	7 100	26.3	2.10
8.0	0.0608	1.0	12.5	5 400	20	1.60
8.0	0.1108	1.0	12.5	3 220	11.9	0.95
8.0	0.4108	1.0	12.5	1 190	4.4	0.35
8.5	0.0055	1.5	12.5	8 730	32.4	2.59
8.5	0.1055	1.5	12.5	3 740	13.8	1.10
8.5	0.2055	1.5	12.5	2 440	9	0.72
8.5	0.4055	1.5	12.5	1 650	6.1	0.49
9.0	0.052	1.5	12.5	3 220	11.9	0.95
9.0	0.102	1.5	12.5	1 970	7.3	0.58
9.0	0.202	1.5	12.5	1 080	4.0	0.32
9.35	0.0128	3.0	12.5	8 450	31.2	2.50
9.35	0.0628	3.0	12.5	5 080	18.8	1.50
9.35	0.1128	3.0	12.5	3 350	12.5	1.00
9.35	0.2128	3.0	12.5	2 580	9.4	0.75
9.5	0.011	3.0	12.5	5 290	19.6	1.55
9.5	0.061	3.0	12.5	2 770	10.3	0.82
9.5	0.111	3.0	12.5	1 860	6.9	0.55
9.5	0.211	3.0	12.5	675	2.5	0.20
9.7	0.0088	3.0	12.5	1 700	6.3	0.50
9.7	0.0588	3.0	12.5	2 100	7.6	0.61
9.7	0.0838	3.0	12.5	970	3.6	0.29
9.7	0.1088	3.0	12.5	1 190	4.4	0.35

* Calculated from the specific activity of dihydroxy[^{14}C]acetone phosphate.
** Moles of dihydroxy[^{14}C]acetone phosphate bound per mole of enzyme.
*** Calculated on the basis of a molecular weight of 159 000 [8].

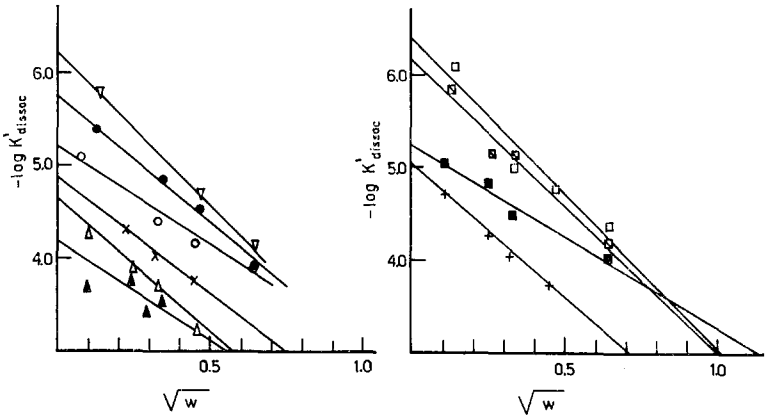

Fig. 1. Plot of $-\log K'_{dissoc}$ of the aldolase–dihydroxyacetone phosphate complex versus $\sqrt{w}$. Data were taken from Table I. ▽, pH 6.0; □, pH 6.5; ▨, pH 7.0; ●, pH 7.5; ■, pH 8.0; ○, pH 8.5; ×, pH 9.0; +, pH 9.35; △, pH 9.5; ▲, pH 9.7.

the value, at each pH, of the thermodynamic dissociation constants (K_{dissoc}), was obtained (Fig. 2).

Effect of temperature on the dissociation constant of the aldolase–dihydroxyacetone phosphate complex

The effect of temperature on the dissociation constant of the aldolase–dihydroxyacetone phosphate complex is illustrated in Fig. 3. ΔG^0 for the reaction of formation of the complex was calculated from the equation: $\Delta G^0 = RT \ln K_{dissoc}$. ΔH^0 calculated from the slope, $\Delta H^0/2.303R$, of the straight line obtained by plotting $-\log K_{dissoc}$ against $1/T$. ΔS^0 was calculated from the equation: $\Delta S^0 = (\Delta H^0 - \Delta G^0)/T$. At 26 °C the following figures were obtained: $\Delta G^0 = -6.58$ kcal·(mole of active site)$^{-1}$; $\Delta H^0 = -2.28$ kcal·(mole of active site)$^{-1}$; $\Delta S^0 = 15.4$ cal·deg^{-1}·(mole of active site)$^{-1}$.

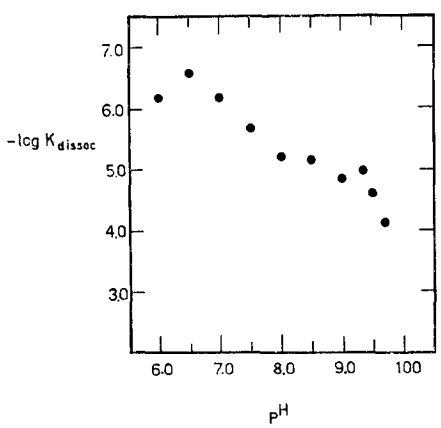

Fig. 2. $-\log K_{dissoc}$ of the aldolase–dihydroxyacetone phosphate complex as a function of pH. The values reported on the figure were obtained by extrapolation to zero ionic strength of the straight lines of Fig. 1 (●).

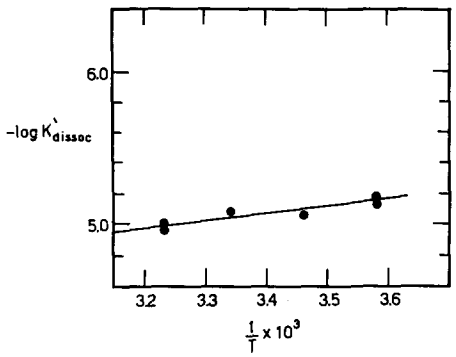

Fig. 3. Effect of the temperature on the $-\log K'_{dissoc}$ of the aldolase-dihydroxyacetone phosphate complex. The mixtures (1 ml), containing aldolase (12.5 nmoles) and 0.05 M Tris–HCl buffer (pH 7.5), adjusted to 0.05 ionic strength by the addition of NaCl; were submitted to gel filtration through Sephadex G-50 columns (1.2 cm × 37 cm) equilibrated with 10 μM dihydroxyacetone phosphate (spec. act. 270 cpm/nmole) and 0.02 M Tris–HCl buffer (pH 7.5), adjusted to 0.05 ionic strength by the addition of NaCl. Temperature was as indicated. The columns were operated as described under Experimental. The fractions collected were analyzed for the radioactivity and the protein content.

DISCUSSION

The thermodynamic data that have been obtained through the study of the reaction of dihydroxyacetone phosphate with aldolase, by means of the gel filtration technique, refer to the sum of the different enzyme–dihydroxyacetone phosphate intermediates (carbinolamine, protonated and non-protonated Schiff base, carbanion). They are thus related not only to the simple binding reaction of the substrate to the enzyme, but also to several steps of the catalytic process leading to the carbanion formation at the C1 of dihydroxyacetone phosphate.

Similar studies had been performed by means of the calorimetric and ultraviolet spectroscopic techniques [12–16]. In these cases, however, the ligands used were not the substrates but phosphorylated polyhydric alcohols, which are inhibitors of the enzyme and do not bind covalently to it.

In agreement with the kinetic data of Mehler and Bloom [17], Mehler [18] and Sanchez de Jimenez et al. [19], we have confirmed that the increase of the ionic strength of the medium increases the dissociation constant of the enzyme–dihydroxyacetone phosphate complex; in our case, however, the effect is that predicted by the limiting form of the Debye–Hückel equation. Our data also show that the interaction of charged groups of the enzyme with the negatively charged substrate is a relevant feature in the formation of the enzyme–substrate complex. Furthermore, they show that the relevant charged groups of the enzyme are positively charged as is apparent from the negative value of the slope ($Z_e Z_s$) of the straight lines obtained by plotting $-\log K'_{dissoc}$ against $\sqrt{w}$.

On the basis of the present knowledge on the structure of aldolase it seems premature to attempt to distinguish whether the effect of ions on the affinity of the substrate and the enzyme is caused by the electrostatic interactions of the added ions with the substrate and the enzyme (primary salt effect) or by changes in the dissociation constants of groups of the substrate and of the enzyme (secondary salt effect). To discriminate between these two possibilities quantitative information on the charge

at the active site of the enzyme, on the ionic radius of the interacting species and on the nature of the active form of the substrate is needed.

The evaluation of the thermodynamic dissociation constants of the dihydroxyacetone phosphate–aldolase complex was performed by extrapolation to zero ionic strength of the experimental values at a set of different ionic strengths. This was done by making use of the limiting form of the Debye–Huckel equation. The dissociation constants increased from $6.3 \cdot 10^{-7}$ M at pH 6.0 to $7 \cdot 10^{-5}$ M at pH 9.7.

The plot of $-\log K_{\text{dissoc}}$ versus pH revealed two downward bends at pH 7.0 and 9.5, and one upward bend at pH 8.0. The slope in the pH 7.0 to 8.0 region was −1.0, that between 8.0 and 9.5 was not integral and not zero and that above pH 9.5 was −2.5, i.e. non-integral. Following the analytical treatment of Dixon [20], the bend at pH 7.0 is assigned to a single set of groups with pK 7.0. These groups could belong either to the enzyme or to dihydroxyacetone phosphate (pK_2 = 6.45) [21]. Work is in progress for the correct settlement of this point. In the region between pH 8.0 and 9.5 the pH dependence is more complex, as is shown by the non-integral slopes of the lines, and cannot be interpreted in terms of only 2 pK values. Rose and O'Connel [22] obtained similar values with the fluorescence quenching technique for the dissociation constant of the aldolase–dihydroxyacetone phosphate complex. The figure of 4.5 μM at pH 7.5 and w = 0.035 (Fig. 2 of the paper of Rose and O'Connel) compares well with the figure of 2.7 μM obtained by us (Fig. 1) under the same experimental conditions of pH and ionic strength.

Our data, however, differ from those of Rose and O'Connel for the pH dependence. We show in fact that the dissociation constant increases sharply above pH 7.0. A partial explanation of this is given in the following. In the paper of Rose and O'Connel the equilibrium constant is calculated from the total and not from the free dihydroxyacetone phosphate concentration which leads to an overestimation of the dissociation constant, especially at the low value of the substrate concentration and the ionic strength is not kept constant, for example in the experiment of Fig. 3 where it decreases by a factor of two between pH 7.0 and 8.0.

The increase of the temperature, between 6 and 36 °C, increases the dissociation constant of the aldolase–dihydroxyacetone phosphate complex. At pH 7.5 and 26 °C, the formation of the complex occurs with a decrease of the enthalpy and an increase of the entropy; the entropy change, however, gives the major contribution to the binding of the substrate.

In a previous report [12] non-linear Arrhenius plots had been obtained which had been interpreted as indicating the existence of at least two conformers of the enzyme or of a large decrease in the apparent heat capacity accompanying the binding of the ligand to a single form of the enzyme [14]. In our experiments we obtain a linear Arrhenius plot. This difference is not at all surprising since we are dealing with equilibrium instead of with kinetic data, and with different reactions (the binding of dihydroxyacetone phosphate instead of D-arabinitol 1,5-bisphosphate, of hexitol 1,6-bisphosphate or of the cleavage of fructose, 1,6-bisphosphate).

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