QUANTITATIVE EVALUATION OF THE CARBANION INTERMEDIATE IN THE ALDOLASE REACTION

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SUMMARY The concentration of the carbanion intermediate formed in the reaction of carboxypeptidase-treated aldolase with dihydroxyacetone phosphate has been measured. This has been done by studying the transient kinetic of the oxidation of the enzyme-substrate complex by hexacyanoferrate (III). The same technique has also been used to titrate the substrate binding site of native aldolase.

The aldolase catalyzed condensation of dihydroxyacetone phosphate with glyceraldehyde 3-phosphate involves the intermediate formation of an enzyme-DHAP carbanion. Evidence for this is based: (a) on the demonstration of the enzyme catalyzed stereospecific exchange with water of the hydrogen atom at the CI of (DHAP) (1-2); and (b) on the susceptibility of the enzyme-DHAP complex to be attacked by tetranitromethane (3,4) and by HCF(III). In the latter reaction 2 moles of HCF(III) oxidize I mole of DHAP yielding 2 moles of HCF(II) and I mole of hydroxypyruvaldehyde (5).

In this paper it will be shown that, by making use of this reaction with carboxypeptidase treated aldolase, the concentration of the enzyme-DHAP carbanion intermediate can be measured. With the same technique the titration of the substrate binding sites of native aldolase can also be performed.

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Abbreviations used are: HCF(III), hexacyanoferrate (III); CBP-aldolase, carboxypeptidase-treated aldolase; DHAP, dihydroxyacetone phosphate; $\Gamma/2$, ionic strength.

EXPERIMENTAL SECTION

Materials - Fructose diphosphate aldolase was prepared from rabbit muscle by the procedure of Taylor et al. (6) and was recrystallized five times. The specific activity of the preparation used in these experiments was 18 units/mg of protein. A unit was defined as the amount which catalyzes the cleavage of I μmole of fructose 1,6-diphosphate per minute under standard assay conditions. Dihydroxyacetone sulfate was prepared as previously described (7). DHAP and carboxypeptidase A (treated with diisopropylfluorophosphate) were purchased from SIGMA Chemical Co. Potassium hexacyanoferrate (III) was obtained from Merck, West Germany.

Methods - Fructose diphosphate aldolase activity was measured in the test system described by Racker (8). Aldolase concentration was determined from the absorbance at 280 mμ considering that the absorbance of 1 mg of pure enzyme/ml (light path 1 cm) is 0.91 (9). The molecular weight aldolase is 159,000 (10). Digestion of aldolase with carboxypeptidase A was performed according to Rutter et al.(11) except that the aldolase/ carboxypeptidase ratio was 70. After digestion, the bulk of carboxy-peptidase was removed according to Spolter et al.(12). The specific activity of CBP-aldolase was 0.32 units/mg of protein.

Stopped-flow measurements were performed with a Durrum D-110 rapid mixing spectrophotometer. The dead time of the instrument was found to be 3 msec. Measurements were made at 420 mμ, the absorption maximum of HCF(III). The molar extinction coefficient was 1,000 cm^{-1} M^{-1}. The light path of the instrument was 2 cm, this was taken into account in the calculations. All the concentrations of the reagents refer to concentrations after mixing in the stopped flow mixing chamber.

The ionic strength ($\Gamma/2$) was kept to 0.05 by the addition of NaCl. The concentration of DHAP used (0.38 mM) allowed more than 99% enzyme saturation (7).

RESULTS

Transient kinetic of the reduction of HCF(III) by the aldolase-DHAP complex - HCF(III) is reduced in the presence of aldolase and DHAP (Fig.1). Independently of the premixing conditions (enzyme and substrate, or HCF(III) and substrate premixed), the time course of the reaction, which presents a rapid and a slow phase, is identical. No reduction occurs, for the time of the experiment, when HCF(III) is mixed either with the enzyme or the substrate alone.

Dihydroxyacetone sulfate that, by reaction with the enzyme forms a Schiff base but does not form the carbanion (7), does not promote the reduction of HCF(III). This provides further eviden-

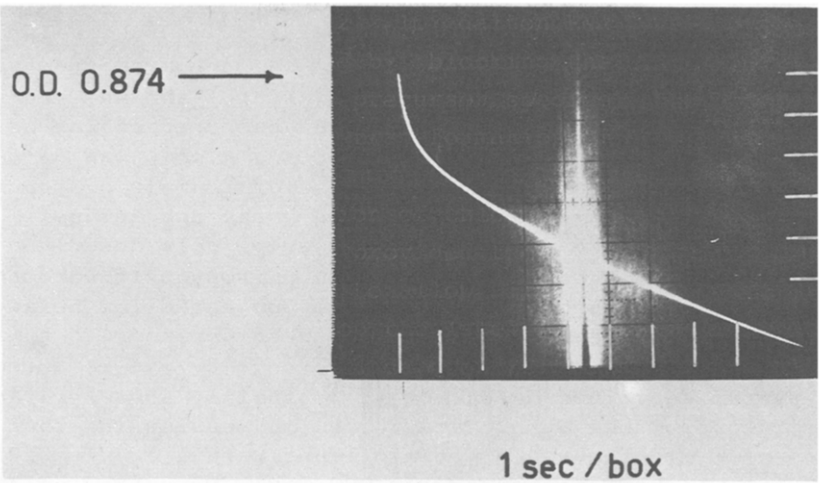


Figure 1) Transient kinetic of HCF(III) reduction by the aldolase-DHAP complex - Enzyme and substrate are premixed. pH 7.0, tris HCl buffer 0.05 M, $\Gamma/2$ = 0.05; Aldolase 4 μM, DHAP 380 μM, HCF(III) 437 μM. Temperature is 24°. Ordinate; ΔOD_{420}= 0.05/box; Abscissa: 1 sec/box.

ce that the carbanion intermediate is indeed the oxidizable species.

The rapid phase of the reaction is of the pseudo first order with respect to both the enzyme-DHAP complex and HCF(III), for the latter this holds up to the concentration of 1 mM. The rapid reaction is therefore of the second order with a rate constant of 7500 M^{-1} sec^{-1} at pH 7.0 and 24°.

The amplitude of the rapid phase is proportional to the concentration of the enzyme (Fig.2).

Transient kinetic of the reduction of HCF(III) by the CBP-aldolase-DHAP complex - With CBP-aldolase, the progress of the reaction with HCF(III) becomes dependent of the premixing conditions. The rapid phase is detectable only if the enzyme and the substrate are premixed (Fig.3). The average amplitude of the rapid phase is about 1/3 of that obtained with the native enzyme (ΔOD_{420} is 0.08 and 0.026 with 6 mμmoles of native and CBP-aldolase respectively).

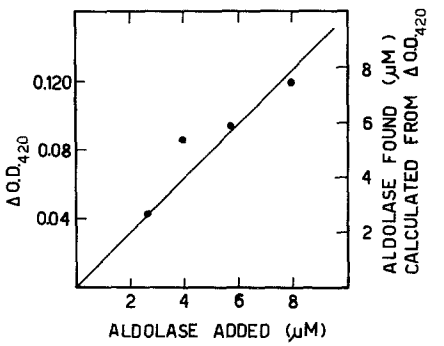


Figure 2) Amplitude of the rapid phase as a function of the aldolase concentration - Enzyme and substrate are premixed. pH 7.0, tris-HCl 0.05 M, $\Gamma/2 = 0.05$; DHAP 380 μM, HCF(III) 437 μM. Aldolase is as indicated. Temperature is 24°.

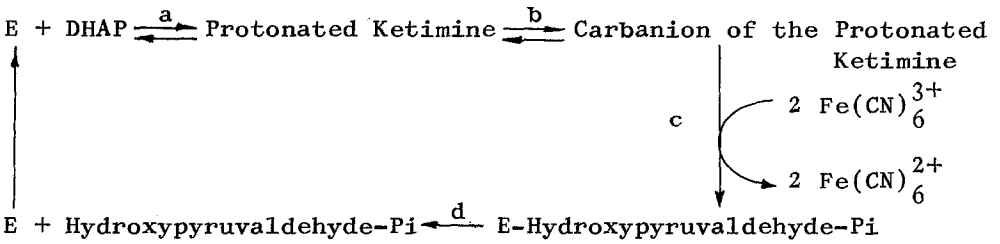
On the left scale of the ordinate is indicated the amplitude of the rapid phase. This has been estimated as a difference between the starting point of the reaction and the point obtained, on the ordinate axis, by linear extrapolation of the slow phase of the reaction to the zero time.

On the right scale of the ordinate is indicated the concentration of aldolase as calculated from the amplitude of the rapid phase. The change in the absorbance per mole of enzyme has been taken as 16×10^3. This on the basis of four substrate binding sites for the enzyme (7) and of the uptake of two molecules of HCF(III) for each site.

The rate constant for the rapid reaction is 8000 $M^{-1} sec^{-1}$ at pH 7.5 and 28°.

DISCUSSION

The reaction of HCF(III) with the aldolase-DHAP complex is summarized in the following schema (5, 13):



The presence of a rapid phase in the reduction of HCF(III), also

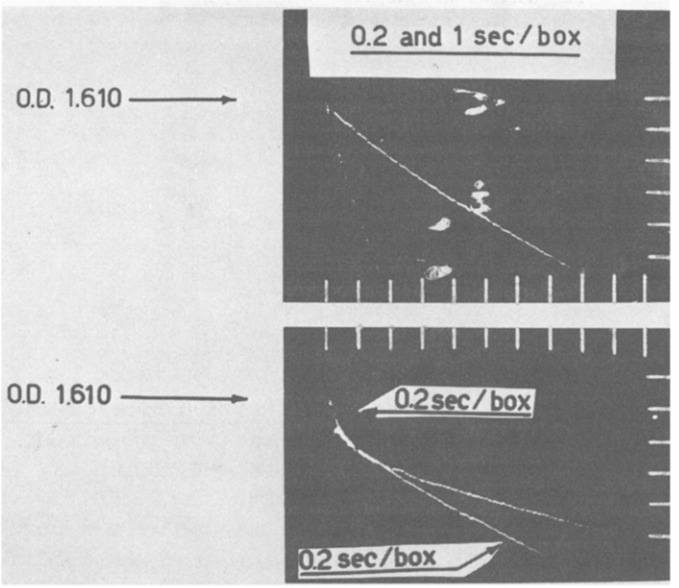


Figure 3) Effect of the premixing conditions in the transient kinetic of HCF(III) reduction by the CBP-aldolase-DHAP complex
HCF(III) and substrate are premixed (upper figure); enzyme and substrate are premixed (lower figure). pH 7.5, tris-HCl buffer 0.05 M, $\Gamma/2 = 0.05$; aldolase 5.8 μM, DHAP 380 μM and HCF(III) 800 μM. Temperature is 28°.
Ordinate: $\Delta OD_{420} = 0.02$/box; Abscissa: 0.2 and 1.0 sec/box (upper figure). 0.1 and 0.2 sec/box (lower figure).

when the enzyme and the substrate have not been premixed, indicates that either step (c) or (d) are the rate limiting steps. The amplitude of the rapid phase does, therefore, reflect the total concentration of the intermediate enzyme-substrate complexes.

With CBP-aldolase a rapid phase is obtained only when the enzyme and the substrate are premixed. This shows that the rate limiting step is now before step (c), and that the amplitude of the rapid phase is now a measure of the concentration of the intermediate enzyme-substrate complexes included between the limiting step and step (c). It has been shown that treatment of aldolase with carboxypeptidase A affects only the rate of the formation of the carbanion (step (b) in the schema)(13). The ampli-

tude of the rapid phase with CBP-aldolase is therefore a measure of the concentration of the enzyme-DHAP carbanion which, in this derivative of aldolase, at pH 7.5 and 28°, represents about 30% of the total intermediates.

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