ON THE SUBSTRATE SPECIFICITY OF FRUCTOSE 1,6-DIPHOSPHATE ALDOLASE (E.C.4.1.2.13) FROM RABBIT MUSCLE: A CRITICAL REVISION.

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SUMMARY The substrate specificity of aldolase has been reinvestigated. It has been found that the enzyme utilizes the keto and the hydrated forms of dihydroxyacetone phosphate. It has been also proposed that not only β-D-fructofuranose 1,6-diphosphate, but also α-D-fructofuranose 1,6-diphosphate is a substrate for aldolase.

The investigation on the substrate specificity of fructose diphosphate aldolase was largely stimulated by the studies on the tautomeric composition of the water solution of fructose 1,6-diphosphate (FDP) (1,2,3) and of the hydrated-keto equilibrium of dihydroxyacetone phosphate (DHAP) (1,4). It was proposed that the enzyme accepts β-D-fructofuranose 1,6-diphosphate (5) and the keto form of DHAP as the active substrates (4). In a conflicting report it was, on the contrary, argued that aldolase binds all the forms of FDP present in solution (6).

We have reinvestigated the problem by taking advantage of the susceptibility of the aldolase-DHAP carbanion intermediate to be oxidized by HCF(III) (7). By this technique we have already shown that the substrate binding sites of aldolase are all catalitically active (8). We now report evidences that aldolase acts on the keto as well as on the hydrated forms of DHAP; and that the enzyme utilizes not only β-fructofuranose 1,6-diphosphate but, probably, also α-fructofuranose 1,6-diphosphate.

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Abbreviation used are: DHAP, dihydroxyacetone phosphate; FDP, fructose 1,6-diphosphate; HCF(III), hexacyanoferrate (III).

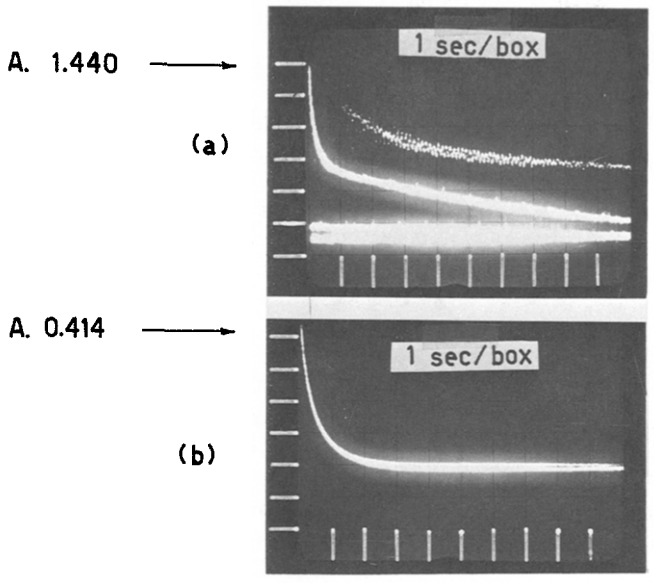


Fig.1) Transient kinetic of HCF(III) reduction in the presence of aldolase and either DHAP or FDP.- (a) HCF(III) and DHAP are premixed. pH 7.0, tris-HCl buffer 5 mM; ionic strength 0.013. Aldolase 53.2 μN, DHAP 17 μM and HCF(III) 720 μM. Temperature is 4°. (b) HCF(III) and FDP are premixed. pH 7.0, tris-HCl 5 mM, ionic strength 0.013. Aldolase 62 μN, FDP 20 μM and HCF(III) 207 μM. Temperature is 26°.
Ordinate: A_{420}= 0.01/box; Abscissa: 1 sec/box.

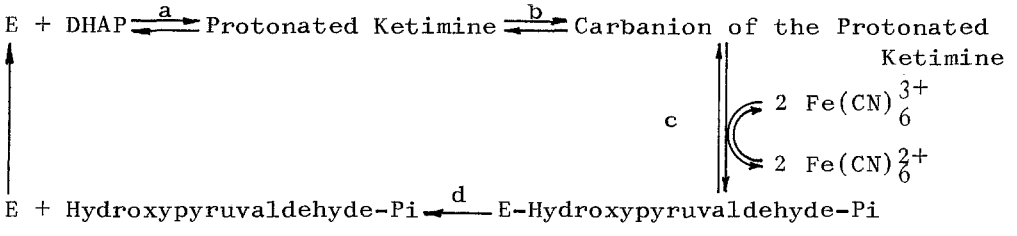
EXPERIMENTAL SECTION

Methods - 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}. 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 concentration after mixing in the stopped flow mixing chamber. The ionic strength was kept to 0.013 by the addition of NaCl.
Other methods and material were as previously described (8).

RESULTS

HCF(III) is rapidly reduced by either DHAP or FDP in the presence of aldolase. This is due to the formation of the aldolase-DHAP carbanion intermediate which is oxidized by HCF(III)(7). The limiting step is the release of the hydroxypyruvaldehyde phosphate from

the enzyme (8). This is also the only irreversible step in the sequence, since, as it will be shown later, the oxidation of the enzyme-DHAP complex appears to be an equilibrium reaction.



Provided aldolase is used in excess over the substrate, the system is suitable to investigate the specificity of the enzyme for the different forms of the substrates existing in solution. Excess aldolase (53.2 μN)(four substrate binding sites per molecule) was thus mixed, at 4°, with DHAP (17 μM) and HCF(III)(720 μM), and the reduction of HCF(III) was followed. The reaction showed a rapid and a slow phase. The amplitude of the rapid phase was 55% of the total change in the absorbance (Fig.1a). Since, at 4°, the relative concentration of the keto form of DHAP is only 17% (4), and the rate observed for the rapid phase (4.4 sec^{-1}) is much larger that the rate of the conversion of the hydrated to the keto form of DHAP (0.44 sec^{-1} at 20°) (4), it was concluded that both the keto and the hydrated forms of DHAP are substrates for the enzyme.

The rapid phase is limited to 55% of the total change in the absorbance because further oxidation of the carbanion intermediate is dependent of the slow recycling of the enzyme (step d), step (c) being a reversible step. It was in fact shown that the amplitude of the rapid phase increases with HCF(III) concentration. Of these experiments are reported those performed at 26° by mixing aldolase (62 μN), FDP (20 μM) and HCF(III) (207 μM). The reaction showed two phases, a rapid and a slow one (Fig.1b). By increasing the concentration of HCF(III), not only the rate of the rapid phase was increased, but also its amplitude (Fig.2) and the rate of the slow phase (Table I). This is as expected for step (b) being reversible.

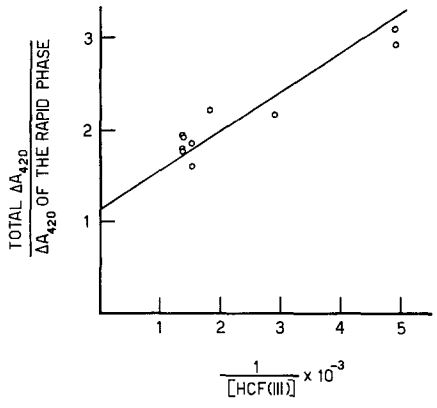


Fig.2) Amplitude of the rapid phase as a function of HCF(III) concentration.- Experimental conditions are the same as in Fig.1b. HCF(III) concentration is as indicated in the figure. The amplitude of the rapid phase has been estimated as the difference between the starting point of the reaction and the intersection with the ordinate axis of the regression line of the semilogarithmic plot of the slow phase of the reaction. The straight line in the figure has been calculated by the least square method.

TABLE I

Effect of HCF(III) concentration on the rate of the oxidation reaction.

HCF(III) μM	K_{obs} (slow phase) sec^{-1}	K_{obs} (rapid phase) sec^{-1}
205	1.29	5.06
346	1.39	6.14
546	1.57	6.9
745	1.81	8.9

Experimental conditions are the same as in Fig.1b. HCF(III) concentration is reported in the table.

It must be added that, at infinite HCF(III) concentration, the relative amplitude of the rapid phase is between 70 and 110% of the total amplitude. This confirms that β-fructofuranose diphosphate, which represents about 80% of the total forms present in solution (2,3), is a substrate of aldolase, but do not exclude

that also α-fructofuranose 1,6-diphosphate is directly utilized by the enzyme.

It is known that FDP, in the presence of excess aldolase, triose phosphate isomerase, α-glycerolphosphate dehydrogenase and NADH, is rapidly utilized for about 74% while the remaining 26% is utilized more slowly. This was taken as a proof that only β-fructofuranose 1,6-diphosphate is a substrate for aldolase, the slow phase being attributed to the slow, spontaneous anomerization of the α to the β form(5). If, however, DHAP is released from aldolase both in the keto and in the hydrated forms, the slow phase can also be explained by the slow, spontaneous conversion of DHAP from the hydrated to the keto form, the only one utilized by α-glycerophosphate dehydrogenase (4). This interpretation, of course, excludes any anomeric specificity of aldolase for FDP.

It was also reported that aldolase binds only the keto form of DHAP (4). This conclusion was derived from experiments of competition for DHAP between aldolase and α-glycerophosphate dehydrogenase. These experiments proved that aldolase binds the keto form of DHAP but not at all that this was the only form bound. In fact, as it is shown in the present report, both the hydrated and the keto form of DHAP are substrates of aldolase.

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