ANOMERIC SPECIFICITY OF FRUCTOSEDIPHOSPHATE ALDOLASE E.C.4.1.2.13 FROM RABBIT MUSCLE

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Summary: Fructosediphosphate aldolase from rabbit muscle is shown to accept ß-D-fructofuranose-1,6-diphosphate as substrate, whereas α-D-fructofuranose-1,6-diphosphate can only be cleaved by the enzyme after a spontaneous change of configuration. The first order velocity constant of the spontaneous reaction was computed to be 0.55 sec^{-1} (at 25° C, pH 7.6). The equilibrium mixture of D-fructose-1,6-diphosphate was computed to 26% α- and 74% ß-D-fructofuranose-1,6-diphosphate.

In aqueous solution D-fructose-1,6-diphosphate occurs as an equilibrium mixture of approximately 10 - 20% α-D-fructofuranose-1,6-diphosphate and 80 - 90% ß-D-fructofuranose-1,6-diphosphate (1, 2). The keto form and the hydrated keto form of D-fructose-1,6-diphosphate do not exist in solution to an appreciable extent (3).

The multiplicity of configurations of D-fructose-1,6-diphosphate prompts the question whether fructosediphosphate aldolase (E.C.4.1.2.13) is specific with respect to one configuration or is nonspecific. Since fructosediphosphate aldolase cleaves a number of ketosephosphates (4-6) which exist only in the open chain configuration, it seems likely that the enzyme catalyzes the cleavage of the keto form of D-fructose-1,6-diphosphate. However, no direct evidence has been presented, whether one or both furanose forms of D-fructose-1,6-diphosphate are used as substrates by the enzyme (5, 7, 8).

This paper describes stopped flow experiments which indicate, that most likely the enzyme uses ß-D-fructofuranose-1,6-diphosphate as substrate.

Material and methods:

All chemicals of p.a. grade were purchased from E. Merck AG, Darmstadt. D-fructose-1,6-diphosphate, dihydroxyacetone phosphate (dimethylketal), D, L-glyceraldehyde-3-phosphate (diethylacetal), D-glucose-6-phosphate, D-fructose-6-phosphate, NADH, $NADP^+$, fructosediphosphate aldolase, triosephosphate isomerase and glycerol-3-phosphate dehydrogenase, all three enzymes from rabbit muscle, and glucose-6-phosphate dehydrogenase and glucosephosphate isomerase, both enzymes from yeast, as well as Precinorm S were bought from Boehringer Mannheim GmbH. Glucose-6-phosphate 1-epimerase from bakers' yeast (9) was prepared according to ref. (1o).

Protein was determined by the Biuret reaction (11) using Precinorm S as a standard. The enzymes were freed from ammonium sulphate by dialysis. The determination of substrate concentrations and enzyme activities were performed at 25° C in standard buffer pH 7.6 (5o mM imidazole/HCl, 5o mM KCl, 8 mM $MgSO_4$, 2 mM mercaptoethanol) using an Eppendorf photometer. With minor modifications substrates were assayed according to ref. (12). Methods for enzyme activity determinations were taken from ref. (13); modified conditions were: Fructosediphosphate aldolase: 2 mM D-fructose-1,6-diphosphate, o.25 mM NADH, 37o U/ml triosephosphate isomerase and 19 U/ml glycerol-3-phosphate dehydrogenase. Triosephosphate isomerase: 1.35 mM D, L-glyceraldehyde-3-phosphate, o.25 mM NADH, 19 U/ml glycerol-3-phosphate dehydrogenase. Glycerol-3-phosphate dehydrogenase: 1.6 mM dihydroxyacetone phosphate, o.25 mM NADH.

Specific activities were: Fructosediphosphate aldolase 9.5 U/mg, triosephosphate isomerase 37oo U/mg, glycerol-3-phosphate dehydrogenase 19o U/mg.

Activities of glucose-6-phosphate dehydrogenase and glucosephosphate isomerase were determined according to ref. (14). The activity constant of glucose-6-phosphate 1-epimerase was determined in system 2 as described in ref. (9).

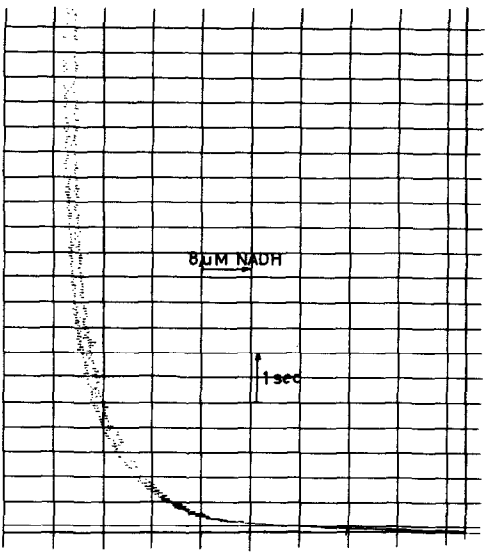


Fig. 1: Record of a stopped flow experiment in the fructosediphosphate aldolase/triosephosphate isomerase/glycerol-3-phosphate dehydrogenase system. 6o U/ml fructosediphosphate aldolase, 57oo U/ml triosephosphate isomerase, 73o U/ml glycerol-3-phosphate dehydrogenase, 32 µM D-fructose-1,6-diphosphate and 8o µM NADH. The reaction is recorded until it is complete. NADH concentration decreases from right to left.

For stopped flow experiments the equipment developed by Hess et al. (15) was used with the monochromator set at 366 nm. The flow system of the apparatus has a total dead time of 2 msec, flow velocity 5 m/sec, volume of the flow chamber 22 µl, d=1 cm. Reactions were initiated by mixing equal volumes of fructosediphosphate aldolase + triosephosphate isomerase + glycerol-3-phosphate dehydrogenase + NADH and D-fructose-1,6-diphosphate + NADH in the respective drive syringes. Final conditions: fructosediphosphate aldolase 1o, 25 and 6o U/ml, respectively, triosephosphate isomerase 57oo U/ml, glycerol-3-phosphate dehydrogenase 73o U/ml, 3o µM D-fructose-1,6-diphosphate and 8o µM NADH.

Results

In order to analyse the reactivity of the various configurations of D-fructose-1,6-diphosphate the fructosediphosphate al-

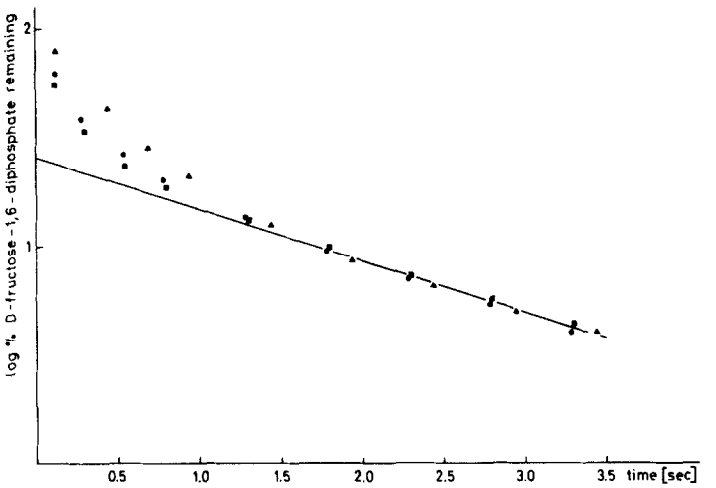


Fig. 2: Semilogarithmic plot of the progress curves of the fructosediphosphate aldolase reaction at three different aldolase activities. 3o μM D-fructose-1,6-diphosphate, 8o μM NADH, 57oo U/ml triosephosphate isomerase, 73o U/ml glycerol-3-phosphate dehydrogenase, (▲) 1o U/ml fructosediphosphate aldolase, (●) 25 U/ml fructosediphosphate aldolase, (■) 6o U/ml fructosediphosphate aldolase. Log % D-fructose-1,6-diphosphate remaining is plotted against the time.

dolase reaction was followed at small concentrations of D-fructose-1,6-diphosphate in the presence of high enzyme activity. Furthermore, activities of triosephosphate isomerase and glycerol-3-phosphate dehydrogenase were used which are high compared with the velocity of the fructosediphosphate aldolase reaction. Thus, the concentrations of the intermediates D-glyceraldehyde-3-phosphate and dihydroxyacetone phosphate become negligibly small and the oxidation of NADH in the glycerol-3-phosphate dehydrogenase reaction is a direct measure of the D-fructose-1,6-diphosphate cleavage in the aldolase reaction.

Figure 1 demonstrates a record of a stopped flow experiment in the fructosediphosphate aldolase/triosephosphate isomerase/glycerol-3-phosphate dehydrogenase system. The course of the

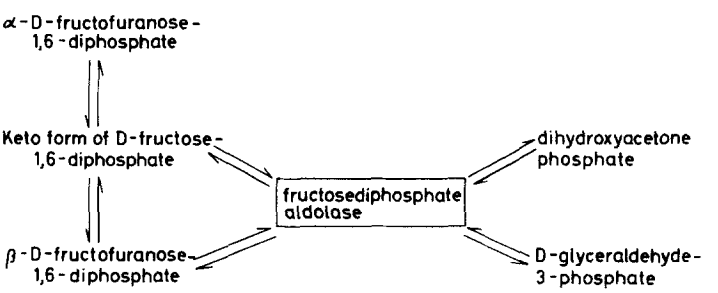
reaction is biphasic: a fast initial reaction is followed by a slower reaction. In figure 2 the progress curves of three reactions of this type at three different activities of fructose-diphosphate aldolase are presented in a semilogarithmic plot. At increasing aldolase activities the fast initial reaction is accelerated, whereas the succeeding slower reaction of first order is not influenced by the aldolase activity. From these experiments it can be concluded that the enzyme in the first phase of the reaction course rapidly reacts with one configuration of D-fructose-1,6-diphosphate, whereas the second phase is determined by a spontaneous conversion of another configuration into the one accepted by the enzyme. From the slope of the regression line for this spontaneous reaction a first order velocity constant of o.55 sec^{-1} is calculated. Extrapolating to zero time the intersection of the regression line with the ordinate reveals that approximately 74% of the equilibrium mixture of D-fructose-1,6-diphosphate react in the fast inital reaction catalysed by aldolase, whereas 26% of the substrate can only be cleaved by aldolase after spontaneous alteration of its configuration.

In order to test whether the spontaneous reaction is enhanced by enzymes which catalyse anomerisation reactions of D-glucose-6-phosphate and D-fructose-6-phosphate, glucosephosphate isomerase from yeast (2oo U/ml) (14,16) as well as glucose-6-phosphate 1-epimerase (1ooo min^{-1} x ml^{-1}) (9, 1o) were added to the reaction mixture. Both enzymes did not cause an alteration of the progress curves excluding a catalysis of the anomerisation of D-fructose-1,6-diphosphate.

Discussion

Our experiments show that fructosediphosphate aldolase re-

acts with 74% of the equilibrium mixture of D-fructose-1,6-diphosphate, whereas about 26% of the substrate can only be cleaved by the enzyme after a spontaneous alteration of its configuration. In NMR experiments (1, 2) it has been found that in aqueous solution D-fructose-1,6-diphosphate exists approximately to 1o% α- and 9o% ß-D-fructofuranose-1,6-diphosphate (1), respectively 2o ± 4% and 8o ± 1o% ß-D-fructofuranose-1,6-diphosphate (2). Therefore, we conclude that aldolase most likely accepts ß-D-fructofuranose-1,6-diphosphate and not α-D-fructofuranose-1,6-diphosphate as substrate. Since the enzyme catalyzes the cleavage of some ketosephosphates which can exist only in the open chain configuration (4-6), the slow reaction observed in our experiments and not being influenced by aldolase, represents most likely the spontaneous ring opening of α-D-fructofuranose-1,6-diphosphate yielding the keto form of D-fructose-1,6-diphosphate which is accepted by the enzyme. Thus, the spontaneous and aldolase catalyzed reactions can be summarized in the following scheme.



Hartmann and Barker (8) were able to show that fructosediphosphate aldolase is competively inhibited by 2,5-anhydro-D-glucitol-1,6-diphosphate and 2,5 anhydro-D-mannitol-1,6-diphosphate, which are structural analogs of α- and ß-D-fructofura-

nose-1,6-diphosphate, respectively. While it seems clear from their data that α- and ß-D-fructofuranose-1,6-diphosphate should bind to the substrate site of aldolase, their argument that α- and ß-D-fructofuranose-1,6-diphosphate should be substrates of aldolase cannot be supported (7).

Finally, we would like to point to the relevance of the anomeric specificity of fructosediphosphate aldolase with respect to the anomeric specificity of its predecessor enzyme in the glycolytic chain. Since fructose-6-phosphate kinase specifically catalyzes the phosphorylation of ß-D-fructofuranose-6-phosphate (17), yielding ß-D-fructofuranose-1,6-diphosphate, which is the furanose configuration of D-fructose-1,6-diphosphate that can be accepted as substrate by fructosediphosphate aldolase, enzyme-catalyzed anomerisation of D-fructose-1,6-diphosphate does not seem to be necessary in the glycolytic pathway.

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