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FRUCTOSE-1,6-BISPHOSPHATE ALDOLASE FROM RABBIT MUSCLE. HALF OF THE SITES REACTIVITY AT LOW TEMPERATURE

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SUMMARY: At -11°C, -13°C only two of the four dihydroxyacetone phosphate binding sites of aldolase are catalytically active. The substrate is very tightly bound to the two sites since it does not exchange with the free substrate of the medium.

The equivalence of the aldolase (EC 4.1.2.13) binding sites was repeatedly demonstrated (1, 2, 3) and there is no evidence of changes of the number of the binding sites between 6 and 36°C (3). A transition of the enzyme was reported to occur at approximately 25°C. This transition affects V_{max} and produces a form which can lower the activation energy of the reaction (4). These same phenomena were also explained by the assumption of a non vanishing ΔC_p or $\Delta C_p^{\ddagger}$ (5).

The temperature dependence of the crystalline forms of aldolase was described and in some cases also the transformation of the low temperature crystals (bipyramidal) into the high temperature crystals (losange shaped) was obtained (6, 7).

We report here a further effect of temperature change on the aldolase reaction. When the temperature is lowered to -13°C only two out of the four catalytic sites of the enzyme remain active, the substrate being very tightly bound to these sites.

MATERIAL AND METHODS. The experiments were performed in 30% (v/v ethylene glycol solution). At these concentrations ethylene glycol appears to have little effect on protein conformation and does not specifically interact with the chromophores of the protein (8). Neither the dissociation constant nor the stoichiometry of ligand binding to aldolase are affected by the presence of ethylene glycol (9). In separate experiments it was shown that ethylene glycol at the concentration employed does not

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inhibit the aldolase reaction. Dihydroxy-[^{14}C] acetone phosphate was prepared from commercial [^{14}C] fructose 1,6-bisphosphate following the procedure of Horecker et al (10) as modified by Ginsburg and Mehler (2).
pH* in ethylene glycol solution was measured by an Ingold LoT 401-TT electrode, standardization was performed according to (11). All the remaining experimental procedures were performed as described (12).

RESULTS AND DISCUSSION. By measuring the aldolase-catalysed exchange of the 3S hydrogen of dihydroxyacetone phosphate it was found that, when the temperature is sufficiently lowered, the release of tritium from the tritiated substrate takes place in a biphasic fashion. In the first phase, independently of the concentration of aldolase, tritium is released in a concentration not exceeding about half the concentration of the substrate binding sites of the enzyme (Fig. 1). In the second phase, between the 2th and the 60th second of the reaction, the rate of the tritium release decreases with the temperature, being 29, 9.5 and 5.6 picoatoms of tritium/nmol of aldolase subunits at -1°C, -4°C and -8°C respectively and pH* 5.2. At -11°C, -13°C no tritium release is detectable after the first phase. The phenomenon is reversible. Aldolase, preincubated at -13°C for a few minutes in the presence of dihydroxyacetone phosphate, displays the same catalytic activity as untreated aldolase when warmed again at room temperature.

According to the widely accepted mechanism of the aldolase reaction (eq. 1)

$$E + AH \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EAH \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E\begin{matrix}A^- \\ H^+\end{matrix} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EA^- + H^+ \qquad \text{(eq. 1)}$$

the phenomenon we have described can be explained by the following mechanisms:

a) all 4 binding sites of aldolase are catalytically active and $k_3 = 0$, $k_2 = k_{-2}$

In this case the tritium ion does not exchange with the medium and, since $[EAH] = \left[E\begin{matrix}A^- \\ H^+\end{matrix}\right]$ the quenching of the reaction with

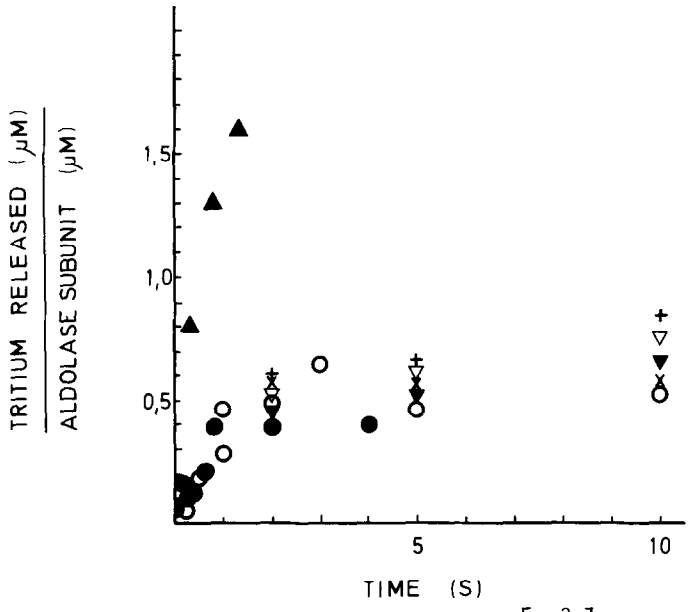

Fig. 1) Release of tritium from the aldolase-[3-^{3}H] dihydroxyacetone phosphate complex as a function of pH and temperature. The incubation mixtures contained:

50 μM aldolase subunit, 0.2 mM [3-^{3}H] dihydroxyacetone phosphate specific radioactivity 100 cpm/nmol, 10 mM acetate buffer, 30% ethyleneglycol; temperature was -12°C, pH* was 5.28 (●).

30 μM aldolase subunit, 0.25 mM [3-^{3}H] dihydroxyacetone phosphate, 10 mM tris-HCl buffer, 30% ethyleneglycol, temperature was -11°C, pH* 8.33 (○).

12 μM aldolase subunit, 0.1 mM [3-^{3}H] dihydroxyacetone phosphate, 10 mM acetate buffer, 30% ethylene glycol, pH* was 5.2. Temperature was -1°C (+); -4°C (▽); -8°C (▼); -12°C (x).

25 μM aldolase subunit, 0.2 mM [3-^{3}H] dihydroxyacetone phosphate 10 mM acetate buffer, 30% ethyleneglycol, temperature was 18°C, pH* was 5.0 (▲).

These experiments were performed with a Durrum multimixer apparatus. The release of tritium was determined as in (12). Data were not corrected for isotopic dilution.

trichloroacetic acid releases tritium only from half of the molecules of substrate bound to the enzyme.

b) only 2 of the 4 binding sites of aldolase are catalytically active.

To distinguish between these two possibilities the binding of dihydroxyacetone phosphate to aldolase was investigated by studying the partition of the substrate between the enzyme and an anion exchange resin (Dowex 1x8). In preliminarly experiments it was found that, under our conditions, the enzyme was not absorbed by the resin and that, provided that the substrate

TABLE I

The exchange of dihydroxyacetone phosphate bound to aldolase with the dihydroxyacetone phosphate in the medium

	Total Radioactivity	Radioactivity in the supernatant solution after the addition of the resin	
		Sample without aldolase	Sample with aldolase
	cpm/ml	cpm/ml	cpm/ml
Labelled substrate added first	9,000	1,900	3,750
Unlabelled substrate added first	9,000	1,800	1,700

The complete incubation mixtures contained 25 μM aldolase subunit, 75 μM dihydroxyacetone phosphate, 75 μM [^{14}C]dihydroxyacetone phosphate (specific radioactivity 120 cpm/nmol), 30% (v/v) ethylene glycol and 10 mM acetate buffer. pH* was 5.28, temperature was -13°C. In one set of experiments labelled substrate was added first followed, after 30 s, by the unlabelled substrate and after 30 s by 300 mg of Dowex 1x8 Cl^- (100-200 mesh) resin equilibrated at-13°C with 30% ethylene glycol and 10 mM acetate buffer, pH* 5.28. After further 30 s of incubation the solutions were separated from the resin by aspiration by mean of a plastic syringe equiped at the bottom end with a disk of spontex. In a second set of experiments, unlabelled substrate was added first followed by the labelled substrate. Control samples without aldolase were also analyzed. Radioactivity was then determined as described in (12).

([^{14}C] dihydroxyacetone phosphate) was in a suitable excess, the enzyme was able to elute from the resin an amount of substrate roughly proportional to the amount of enzyme added. We then investigated the exchange of unlabelled dihydroxyacetone phosphate bound to the enzyme with [^{14}C] dihydroxyacetone phosphate and viceversa. As shown in Table I when, at pH* 5.28 and -13°C, to 25 μM aldolase subunit was added 75 μM [^{14}C] dihydroxyacetone phosphate, followed after 30 s by 75 μM unlabelled dihydroxyacetone phosphate and than after additional 30 s by the resin, in the supernatant solution were found 3,750 cpm/ml, as compared to 1,900 cpm/ml found in a control sample without aldolase. In the reverse experiment, however, where the unlabelled substrate was added first, followed by the labelled substrate, no significant difference of radioactivity was

found in the supernatant solution of the sample with aldolase as compared to the sample without aldolase.

The result of this experiment shows clearly that at -13°C, even after an incubation of 30 s, the exchange between the substrate bound to the enzyme and the substrate free in the medium is still undetectable.

Since the radioactive substrate bound to the enzyme does not exchange with the substrate of the medium in the time scale of the experiment, the amount of substrate bound to the enzyme can be calculated from the experiment of Table I, where labelled substrate was added first, by the formula:

$$\frac{\text{sample with aldolase (cpm)} - \text{sample without aldolase (cpm)}}{\text{specific radioactivity of the substrate (cpm/ml)}} =$$

$$= \frac{3,750 - 1,900}{120} = 15.4\ \mu M,$$ which represents 61% of the aldolase binding sites. These experiments, together with those described in Figure 1, provide evidence that only 2 out of the 4 binding sites of aldolase are catalytically active at low temperature and that the substrate is very tightly bound to these two sites. The phenomena so far described are certainly due to the temperature change since we have previously shown that all the four aldolase binding sites are catalytically active between pH 5.0 and 9.0 for temperatures between 4° and 24°C (13, 14).

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