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Inhibition of Transketolase by Hexacyanoferrate(III)

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Abstract—The effect of hexacyanoferrate(III) on the catalytic activity of transketolase has been studied. This oxidant inactivates only one of two active sites of the enzyme, the one with a higher affinity to the coenzyme (thiamine diphosphate). The second active site does not lose its catalytic activity. These observations indicate that the active sites of holotransketolase, being indiscernible by data of X-ray analysis, exhibit functional nonequivalence.

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Transketolase (EC 2.2.1.1), one of the key enzymes of the pentose phosphate pathway of carbohydrate metabolism, is a thiamine diphosphate-dependent enzyme. Together with transaldolase, this enzyme provides communication between the pentosophosphate pathway and glycolysis [1]. Transketolase (TK) is found in all investigated organs and tissues of animals or plants as well as in microorganisms [2]. It exhibits wide substrate specificity and catalyzes the two-substrate reaction of reversible transfer of a two-carbon fragment (glycolaldehyde residue) from keto sugars (donor substrates) to aldo sugars (acceptor substrates). The typical physiological substrates of transketolase are xylulose-5-phosphate, fructose-6-phosphate, sedoheptulose-7-phosphate (donor substrates) as well as ribose-5-phosphate and glyceraldehyde-3-phosphate (acceptor substrates). Besides thiamine diphosphate, bivalent cations (magnesium, calcium, manganese) serve as cofactors of the enzyme [3]. The most investigated enzyme is the transketolase from *Saccharomyces cerevisiae*.

The enzyme is a homodimer with a molecular weight of 148.4 kDa. The enzyme has two active centers with identical structures (by crystal X-ray structure analysis), exhibiting equal catalytic activity [4-7]. The native holoenzyme contains Ca^{2+} (2 g-atoms per mol protein). It takes part in the formation of the active site, serving as a bridge between the coenzyme and the apoenzyme [8, 9]. The substitution of Ca^{2+} by Mg^{2+} does not affect the catalytic activity of the enzyme, but it alters the structure of the protein molecule and the affinity of thiamine diphosphate to the apoenzyme [10]. The data of the X-ray analysis and site-directed mutagenesis allowed identification of functional groups involved in the interaction of apotransketolase with the coenzyme and responsible for substrate binding and catalysis [11].

In spite of significant achievements in the investigation of transketolase, some problems remain little explored, for example, the regulation of the enzyme activity, influence of activators and inhibitors, etc. Investigation of these problems would help in understanding of functional features of the enzyme. One of the few works in this field is that of Christen et al. [12], where the effect of hexacyanoferrate(III) on transketolase activity was studied. They demonstrated that in the presence of this oxidant the holotransketolase is rapidly and irreversibly inactivated by 50%, and then the enzyme maintains its residual activity during subsequent period of observation. These data suggest that since the active sites of transketolase do not differ in catalytic activity [5], the decrease in the activity by 50% must indicate that in the presence of hexacyanoferrate(III) only one of two active sites is inactivated, while the second is resistant to the inhibitor. This would demonstrate that the structurally identical active sites of transketolase (according to X-ray analysis [6, 7]) are functionally nonequivalent. The goal of the present work was to test this suggestion.

MATERIALS AND METHODS

Chemicals. Thiamine diphosphate (TDP), glyceraldehyde-3-phosphate dehydrogenase, NAD^+, ribose-5-

Abbreviations: TK, transketolase; TDP, thiamine diphosphate.
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phosphate, glycylglycine, and $CaCl_2$ were from MP Biomedicals (Germany); sodium arsenate and dithiothreitol were from Fluka (Switzerland); Sephadex G-50 was from Pharmacia (Sweden).

Isolation of transketolase. Apotransketolase (apoTK) was isolated from baker's yeast according to an earlier described procedure [13] and stored at 4°C in an ammonium sulfate solution of 50% saturation, pH 7.6. Before experiments the enzyme was freed from ammonium sulfate using a Sephadex G-50 column. The enzyme was homogeneous (by SDS-PAGE) and exhibited specific activity of 28 U/mg. The concentration of the transketolase (TK) was determined spectrophotometrically using $A^{1\%}_{1cm} = 14.5$ at 280 nm [14].

Transketolase activity. The activity of TK was determined spectrophotometrically (Aminco DW-2000) by the rate of the reduction of NAD^+ in the coupled reaction with glyceraldehyde-3-phosphate dehydrogenase [2]. The reaction mixture (1 ml) contained 50 mM glycylglycine, pH 7.6, 2.5 mM $CaCl_2$, 1 mM sodium arsenate, 3.2 mM dithiothreitol, 0.74 mM NAD^+, 0.1 mM TDP, 4 U of glyceraldehyde-3-phosphate dehydrogenase, 0.5 mM xylulose-5-phosphate, and 0.5 mM ribose-5-phosphate, pH 7.6. The reaction was started by the addition of TK.

Preparation of semi-holoTK1 (the enzyme where only one of two active sites is functioning). The active sites of TK are nonequivalent: the affinity of the coenzyme to one of them (arbitrarily the 1st active site) is rather high, exceeding the TDP affinity to the other active site (arbitrarily the 2nd active site) approximately 10-fold. After the addition of the equimolar concentration of TDP to apoTK (3.3-13.5 μM), the coenzyme completely binds to the 1st active site and does not dissociate during subsequent experiments. To prepare semi-holoTK1, 3.3 μM apoTK was incubated with 3.3 μM TDP and 2.5 mM $CaCl_2$ in 50 mM glycylglycine, pH 7.6, for 30 min at 25°C.

Preparation of semi-holoTK2 (the enzyme where only one of two active sites, the one with the lower affinity to TDP, is functioning). To prepare such an enzyme, it is necessary first to block the 1st active site with the higher affinity to TDP with the inactive analog of the coenzyme oxyTDP (its affinity to the active sites of the enzyme is several times higher than that of the natural coenzyme). The preparation of TK with the blocked 1st active site is supplemented with TDP at concentration that is sufficient for the binding with the 2nd active site, but insufficient for the displacement of the oxyTDP from the 1st active site. To prepare semi-holoTK2, 3.3 μM apoTK was incubated with 3.3 μM oxyTDP and 2.5 mM $CaCl_2$ in 50 mM glycylglycine, pH 7.6, at 25°C for 30 min, and then TDP was added to the final concentration of 100 μM.

Preparation of xylulose-5-phosphate. The sodium salt of xylulose-5-phosphate was prepared enzymatically according to a method described earlier using oxypyruvate and glyceraldehyde-3-phosphate as the substrates and TK from baker's yeast as the enzyme [15].

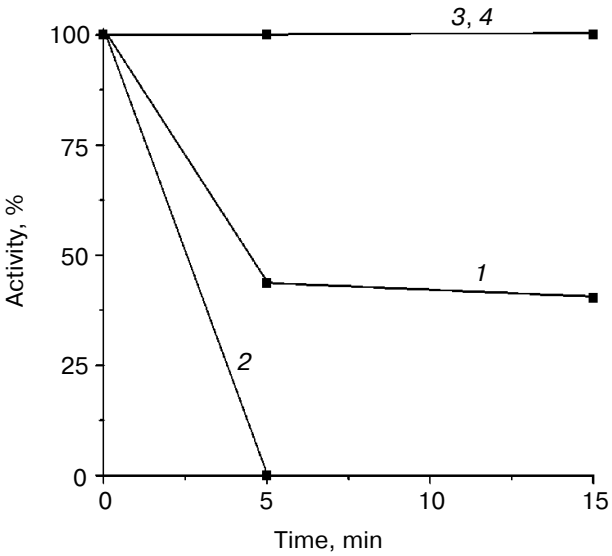
Preparation of holotransketolase. A solution of the apoenzyme (2.1 mg/ml) in 50 mM glycylglycine buffer, pH 7.6, was supplemented with 2.5 mM $CaCl_2$ and 0.1 mM TDP and incubated for 5 min in the cold, then used in the work.

Experimental procedure. Four different forms of transketolase (apoenzyme, holoenzyme, semi-holoTK1, and semi-holoTK2, each in concentration of 0.025 mg/ml) were preincubated in the presence of 0.5 mM $K_3Fe(CN)_6$ in 50 mM glycylglycine buffer, pH 7.6, containing 2.5 mM $CaCl_2$. After different time intervals, aliquots were taken from the incubation mixture to determine the transketolase activity. During the experiment TDP did not dissociate from any of the investigated forms of the holoenzyme.

RESULTS AND DISCUSSION

The figure presents the result of one of several reproducible experiments on the influence of the preincubation of transketolase with hexacyanoferrate(III) on its catalytic activity. The enzyme where only the 1st active site is functioning (the site with the higher affinity to the coenzyme) completely loses its catalytic activity after 5 min of the incubation (curve *2*).

In contrast, the activity of the enzyme with the functioning 2nd active site (with the lower affinity to the coenzyme) does not change during the observation time (curve *3*). Holotransketolase with two functioning active sites is



Effect of hexacyanoferrate(III) on the activity of holotransketolase (*1*), semi-holoTK1 (*2*), semi-holoTK2 (*3*), and apotransketolase (*4*). The enzyme activity before the addition of $K_3Fe(CN)_6$ was taken as 100%

inactivated by 50% after the first 5 min of incubation, and subsequent incubation does not affect the enzyme activity (curve *1*). Thus, hexacyanoferrate(III) inactivates only one of two active sites of transketolase (the one with the higher affinity to TDP), not affecting the second active site. This explains the fact that that the holoenzyme is inactivated only by 50%.

The nature of the inhibiting action of hexacyanoferrate(III) on transketolase remains unclear. However, it is evident that the inhibition is possible only in the case of completely formed active site with the bound coenzyme (apotransketolase is not inhibited by hexacyanoferrate(III), see curve *4*).

The active sites of transketolase work simultaneously, but in phase opposition, i.e. when a ketose cleaves one active site (the first stage of the transketolase reaction), in the other one a ketose forms (the second stage of the transketolase reaction: condensation of the glycolaldehyde residue with the acceptor substrate) [16]. Originally, TDP is likely to be present in one of the active sites in the amino form, and in the other one in the imino form [17].

During the catalysis, these tautomeric forms of the coenzyme pass one into the other [18]. It cannot be excluded that the active sites containing different tautomeric forms of TDP exhibit different sensitivity to the action of hexacyanoferrate(III). This assumption could explain inactivation of one active site and stability of the other.

In any case, the main conclusion is that the structurally identical (according to X-ray analysis) active sites [6, 7] exhibit functional nonequivalence.

It is noteworthy that recombinant human transketolase is inhibited by hexacyanoferrate(III) in the same way (data not shown), although in contrast to the enzyme from *S. cerevisiae* this enzyme exhibits no cooperativity either while binding the coenzyme (TDP) or the substrates (donor and acceptor), or during the catalytic transformation of the substrates.

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