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THE FUNCTIONAL IDENTITY OF THE ACTIVE CENTRES OF TRANSKETOLASE

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Summary

Direct determination of the number of catalytically active molecules of the coenzyme in holotransketolase (sedoheptulose-7-phosphate:D-glyceraldehyde-3-phosphate glycoaldehydetransferase, EC 2.2.1.1) has corroborated our previous data indicating that in the native enzyme there are two active centres. They have been provided to be functionally identical. It has been shown that the decrease in the specific activity of transketolase during its storage is due to inactivation of one of the active centres, having a lower affinity for the coenzyme. The second active centre retains thereby its full catalytic activity.

Introduction

Transketolase (sedoheptulose-7-phosphate:D-glyceraldehyde-3-phosphate glycoaldehydetransferase, EC 2.2.1.1) from baker's yeast has a molecular weight of 159 000 [1,2] and consists of two subunits of the same molecular weight [3,4]. The experimental data obtained previously indicated that a molecule of transketolase contains two active centres [5]. The present paper reports the results of direct determination of the number of the catalytically active molecules of thiamine pyrophosphate in holotransketolase and unambiguous evidence of the functional identity of the active centres of the enzyme.

Materials and Methods

Transketolase was isolated from baker's yeast essentially as described by Racker et al. [6]. Before crystallization, additional fractionation with ammo-

Abbreviation: oxyTPP, oxythiamine pyrophosphate.

nium sulphate was carried out. The precipitate of transketolase obtained as indicated in Ref. 6 was dissolved in cold water to a final concentration of protein of 2 mg/ml. A saturated solution of $(NH_4)_2SO_4$ was added dropwise and fractions were collected in the saturation intervals of 0.55—0.59, 0.59—0.63 and 0.63—0.65. The first fraction was prepared in the cold and the other two at room temperature. The bulk of transketolase was as a rule detected in the 0.59—0.63 saturation fraction. To obtain a homogeneous preparation, the $(NH_4)_2SO_4$ fractionation was repeated once or twice. The enzyme was stored at 2°C in an $(NH_4)_2SO_4$ solution of 0.5 saturation, pH 7.6.

The freshly isolated transketolase had a specific activity of 12 U/mg protein. Apotransketolase was obtained by storing the enzyme preparation (1 mg/ml) in 1.6 M $(NH_4)_2SO_4$, pH 8.4 for 24—48 h.

Before study, the enzyme preparations were passed through Sephadex G-50 equilibrated with 5 mM glycylglycine buffer, pH 7.6. The same buffer was used for elution.

Protein was determined by the method of Lowry et al. [7] and by the value of absorbance at 280 nm, which for a 1% solution of transketolase is 14.5 [8].

The transketolase activity was assayed spectrophotometrically by the rate of reduction of NAD [9]. The composition of the reaction mixture was: 50 mM glycylglycine; 1.1. mM sodium arsenate; 0.37 mM NAD; 0.5 U glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle; 3.2 mM cysteine; 2.3 mM $CaCl_2$; 0.23 mM thiamine pyrophosphate, 10 mM substrate, transketolase. Total volume 2 ml, pH 7.6, 25°C. The mixture of phosphopentoses that was used as a substrate was obtained as described by Gubler et al. [10].

OxyTPP was prepared by phosphorylation of oxythiamine as described in [11]. The mixture of phosphoric esters of oxythiamine was stored in a vacuum exsiccator at +2°C. Shortly before work it was fractionated by preparative high voltage paper electrophoresis in 50 mM sodium acetate buffer, pH 5.4, for 1 h at +8°C. OxyTPP thus obtained was chromatographically pure; its concentration was determined spectrophotometrically [12].

The binding of thiamine pyrophosphate with transketolase was studied spectrophotometrically by the change in the absorbance at 320 nm [5]. The measurements were carried out according to the double-wave scheme at 320 nm and 380 nm in a 'Hitachi-356' spectrophotometer in the following way: 5 mM glycylglycine buffer, pH 7.6, 2.3 mM $CaCl_2$ and apotransketolase (total volume 2.5 ml) were placed in the cuvette. The absorbance of the mixture was measured; then 0.01 ml portions of thiamine pyrophosphate were added. Each portion of thiamine pyrophosphate was added only after the absorbance change due to the addition of the previous portion had ceased.

The quantity of thiamine pyrophosphate that was bound to the protein on formation of holotransketolase was determined in the following way. Apotransketolase was incubated at 20°C with thiamine pyrophosphate and calcium used in saturating concentrations. To remove the free cofactors, the mixture was passed through Sephadex G-50 equilibrated with 5 mM glycylglycine buffer, pH 7.0. The protein-containing fractions were collected and boiled for 5 min at 100°C. The thiamine pyrophosphate cleaved off thereby was determined enzymatically [13].

Results and Discussion

It has been shown previously that between the level of the catalytic activity and the change in absorbance induced by the addition of thiamine pyrophosphate to apotransketolase, there is a clear-cut linear dependence [5]. This means that the catalytic activity is inherent only in those molecules of thiamine pyrophosphate, the interaction of which with the apoenzyme is accompanied by certain changes in the absoption spectrum of the protein.

It should be noted that the association constants for thiamine pyrophosphate in the presence of Ca^{2+} have sufficiently high values, $3 \cdot 10^7$ M^{-1} and $4 \cdot 10^6$ M^{-1} for the first and second coenzyme-binding sites, respectively. Additionally, they differ by one order of magnitude. This allows one, under certain conditions, to titrate with thiamine pyrophosphate the first active center of the enzyme. One of the invariably reproducible experiments is shown in Fig. 1 (curve a). With sufficiently low concentrations of thiamine pyrophosphate, there is a linear correlation between the change in absorbance at 320 nm and the amount of added thiamine pyrophosphate. In addition, it was demonstrated (data not shown) that at different concentrations of apotransketolase, addition of one and the same (sufficiently low) concentration of thiamine pyrophosphate results in the formation of the same quantity of the holoenzyme. It can be concluded therefrom that under these conditions apotransketolase binds the coenzyme almost completely, and the linear part of curve a in Fig. 1 is indicative of the binding of thiamine pyrophosphate in the first active centre that has a greater affinity for the coenzyme than the second centre. On binding of one mole of thiamine pyrophosphate per mole of protein, the change in the absorbance at 320 nm is 50% of the maximal (that is, in the presence of an excess of cofactors). The non-linear part of curve a reflects the binding of the coenzyme in the second active centre. The association constant for thiamine pyrophosphate in the second coenzyme-binding site

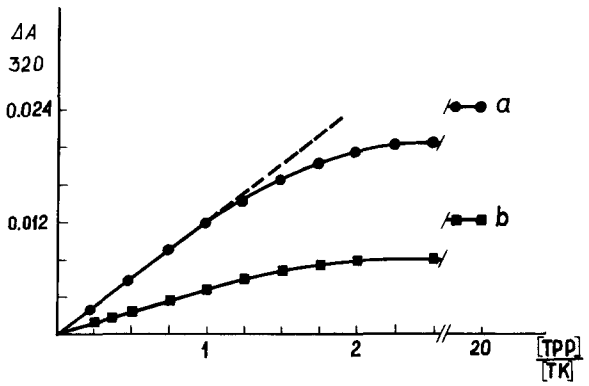

Fig. 1. The binding of thiamine pyrophosphate with native (a) and partially blocked by oxythiamine pyrophosphate (b) apotransketolase. Ordinate, changes in the absorbance at 320 nm; abscissa, number of moles of thiamine pyrophosphate added to the sample per mole of the apoenzyme. Concentration of apotransketoalse, $3.14 \cdot 10^{-6}$ M; of oxythiamine pyrophosphate, $3.14 \cdot 10^{-6}$ M.

determined in these experiments is $4.5 \cdot 10^6$ M^{-1}. This is in good agreement with the figure reported previously and obtained in a different manner [14].

Curve b in Fig. 1 characterizes the binding of thiamine pyrophosphate with apotransketolase in which one of the active centers (that having a greater affinity for the coenzyme) is blocked by oxyTPP *. One can see that this curve has no linear part and it has the same character as the non-linear part of curve a.

The change in the absorbance at 320 nm (see curve b) of such an enzyme and its catalytic activity in the presence of the saturating concentrations of the cofactors is 50% of the maximal that is displayed by native transketolase.

Thus the experimental data obtained may be unambiguously interpreted as indicating that a molecule of transketolase has two active centres that do not differ in the catalytic activity.

Addendum

Inactivation of transketolase during storage

With freshly isolated preparations of the enzyme, the change in the absorbance of apotransketolase at 320 nm observed on the addition to the enzyme of saturating concentrations of the cofactors is always the same, i.e. $6.95 \cdot 10^3$, if calculated per 1 M protein solution. On being stored, the preparations of transketolase gradually lose their specific activity: it decreases from 12 to 9—6 U/mg protein. Thereby the change in the absorbance value of apotransketolase on the formation of the holoenzyme decreases.

Fig. 2 shows the results of titration of freshly isolated and stored apotransketolase by thiamine pyrophosphate in the presence of Ca^{2+}. One can see that the first binding site of the coenzyme is titrated in quite the same manner in both cases. The change in the absorption of apotransketolase at 320 nm and the catalytic activity of the enzyme on the binding of one mole of thiamine pyrophosphate per mole of protein is also the same. This means that the inactivation of transketolase during storage is due to the changes in the properties of the second active centre of the enzyme. Two equally probable reasons for it can be suggested: either inactivation of transketolase induces a decrease in the number of the binding sites of the coenzyme, or their total number is retained but the second active centre has lower catalytic and optical activities.

To answer this question, preparations of the enzyme (fresh and stored) were preincubated with thiamine pyrophosphate and Ca^{2+} taken in saturating concentrations; after that the change in the absorbance at 320 nm was measured and the quantity of thiamine pyrophosphate in the reconstituted preparations of the holoenzyme was determined. As can be inferred from the data listed in Table I, there is a distinct parallelism between these characteristics.

It should then be concluded that decrease in the specific activity of transketolase on storage is to be ascribed to a lower amount of the coenzyme-

* OxyTPP is a nonactive analogue of thiamine pyrophosphate and has a many-fold higher affinity for apotransketolase than thiamine pyrophosphate [15]. On addition of this analogue to apotransketolase in an equimolar concentration, it binds completely with the protein and can be split neither by gel filtration nor after incubation with a 100-fold excess of the coenzyme for 24 h.

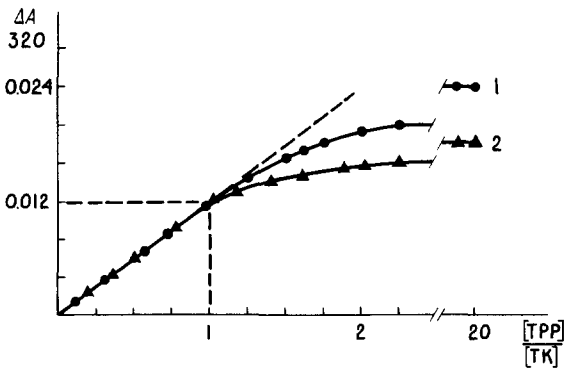

Fig. 2. The binding of thiamine pyrophosphate with freshly isolated (1) and stored (2) apotransketolase. Designations and the concentration of apotransketolase as in Fig. 1.

TABLE I

THE VALUES OF ΔA_{320} AND THE CONTENT OF THIAMINE PYROPHOSPHATE (TPP) FOR STORED PREPARATIONS OF TRANSKETOLASE (TK) AFTER RECONSTITUTION OF THE HOLOENZYME

ΔA_{320} per 1 M protein solution *	Decrease in ΔA_{320} compared to freshly isolated preparations (%)	$\frac{\text{TPP (mol)}}{\text{TK (mol)}}$
$5.55 \cdot 10^3$	20	1.6 (± 0.05)
$4.90 \cdot 10^3$	30	1.4 (± 0.05)

* The value of ΔA_{320} for freshly isolated transketolase is $6.95 \cdot 10^3$.

binding sites in the molecule of the enzyme. The longer the enzyme is stored, the greater amount of transketolase molecules loses its ability to bind the coenzyme in the second active centre. At the same time, the catalytic activity and the optical characteristics of the active centres, which retain the ability to bind the coenzyme, are the same in freshly isolated and stored preparations of transketolase.

The change in the absorbance at 320 nm induced by the interaction of thiamine pyrophosphate and apotransketolase can be used for determining the coenzyme-binding sites in the molecule of the enzyme.

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