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Isolation and Properties of Noncovalent Complex of Transketolase with RNA

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Abstract—A method for isolation of homogenous transketolase from baker's yeast using immunoaffinity chromatography was significantly simplified. It was demonstrated that transketolase could be isolated from fresh yeast in the form of a complex with a high molecular weight RNA. Storage of yeast led to the dissociation of the complex to a low molecular weight complex and then to the free enzyme. Conditions were chosen for complex dissociation and free enzyme isolation. In comparison to the free enzyme, the specific activities of the high and low molecular weight complexes were decreased 20-25- and 3-5.5-fold, respectively. The affinity to the cofactor thiamine diphosphate and to xylulose-5-phosphate (donor substrate) did not change for the low molecular weight complex, while the time of binding to calcium increased. The latter was necessary for the complete manifestation of the enzymatic activity. Changes in the circular dichroism spectrum between 300 and 360 nm after the addition of thiamine diphosphate, which characterize the formation of the catalytically active holoenzyme, were significantly lower for the low molecular weight complex than for the free enzyme.

Key words: transketolase, thiamine diphosphate, RNA, circular dichroism, immunochromatography, baker's yeasts

As demonstrated earlier in our laboratory [1], a noncovalent complex of transketolase (TK) with RNA was released from an immunoaffinity column during isolation of TK from baker's yeasts. This complex had a different absorption spectrum and higher molecular weight with specific activity of about 10% of the activity of the free TK. It was also shown that the relative quantities of the free enzyme and its complex with RNA varied considerably from one experiment to another, but this observation was not studied further. Here we have studied the conditions of isolation, stability, and several properties of the complex.

MATERIALS AND METHODS

The following reagents were used in this work: NAD^+ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from rabbit muscle from Reanal (Hungary); Sepharose 4B, Sephadex G-50, and Sephacryl S-300 from Pharmacia (Sweden); glycerol, xylulose-5-phosphate (X5P), ribose-5-phosphate (sodium salts), hydroxypyruvate, and protamine sulfate from Sigma (USA); thiamine diphosphate (ThDP), dithiothreitol, and glycolaldehyde from Serva (Germany); yeast RNA from Boehringer (Germany); and other reagents from Russian suppliers of "chemically pure" quality. The equilibrated mixture of phosphopentoses, which was used as a substrate for TK, was obtained by the method of Gubler et al. [2].

TK was isolated from baker's yeasts *Saccharomyces cerevisiae*. The extraction was carried out by three methods: autolysis of frozen yeast with toluene [3], autolysis at 43°C [4], and cytolysis of dry yeast with ammonia [3]. The first method was used mainly for the isolation of the high molecular weight complex TK–RNA. The maximal yield of the enzyme was obtained with the third method. The following isolation steps were carried out at 4°C.

TK was purified by immunoaffinity chromatography. To prepare the immunoaffinity column polyclonal antibodies isolated from rabbit serum were immobilized on the BrCN-activated Sepharose 4B. BrCN synthesis, Sepharose activation, and immobilization of antibodies were carried out as described earlier [5]. The optimal level of Sepharose activation was 65 mg of BrCN for 1 ml of precipitated Sepharose, the optimal antibody concentra-

Abbreviations: TK) transketolase; TK-160) low molecular weight complex TK–RNA; TK-210) high molecular weight complex TK–RNA; ThDP) thiamine diphosphate; GAPDH) glyceraldehyde-3-phosphate dehydrogenase; X5P) xylulose-5-phosphate; CD) circular dichroism.

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tion being 6-8 mg for 1 ml of precipitated BrCN-Sepharose. Under these conditions, the immunoaffinity column could function up to 2 years (up to 55 cycles) without loss of its binding capacity.

The binding of yeast extract to the immune column was carried out at pH 7.2. Free nucleic acids in the extract were precipitated before binding to the column by protamine sulfate (not less than 0.7 g for 100 g of dry yeast). The efficiency of TK binding increased from 0.3-0.5 to 1-1.5 mg of enzyme for 1 ml of sorbent after this step, avoiding the need for dialysis and concentration of the protein solution after elution from the immunoaffinity column. Thus, the enzyme was directly precipitated with ammonium sulfate. The nonspecifically bound protein was washed with 300 mM NaCl, 10 mM potassium phosphate buffer, pH 7.2. The bound TK was eluted with 300 mM NaCl, 10% glycerol, 200 mM β-alanine buffer, pH 11.2. Fractions (20 ml) were collected in tubes with 4 ml of 2 M glycine buffer, pH 7.2. Then the fractions that contained protein were combined, the pH was adjusted to 8.8, and the protein was precipitated with dry ammonium sulfate at 65% saturation.

The immunoaffinity column was washed with 150 mM NaCl, 20 mM potassium phosphate buffer, pH 7.2. To obtain a smoother pH gradient (an essential condition for preservation of the binding capacity of the column) a volume of elution buffer (not less than 1/3 of column volume) was overlaid beforehand on the column. The column was stored in the same buffer.

The TK was stored at 4°C in the solution of ammonium sulfate of 0.5 saturation, pH 7.6. For elimination of ammonium sulfate, the enzyme was overlaid before use on a column with Sephadex G-50 that was equilibrated with corresponding buffer. Free (not bound to RNA) enzyme was homogenous according to SDS-PAGE, and had a specific activity of 20 units/mg.

TK concentration was measured according to the method of Bradford [6] and also spectrophotometrically using the absorbance coefficient $A^{1\%}_{1\ cm} = 14.5$ for the free enzyme at 280 nm [7] and 145 at 220 nm for both free TK and its complex with RNA. The error in the protein concentration due to RNA contribution to the absorbance at 220 nm did not exceed 10% for the high molecular weight complex and 2% for the low molecular weight complex (data not shown).

ApoTK was isolated by incubation of the enzyme (1-2 mg/ml) in 1.6 M solution of ammonium sulfate, pH 7.6, for 24-48 h [8].

TK activity was measured spectrophotometrically at 25°C by the velocity of NAD^+ reduction in the system conjugated with GAPDH [9]. The reaction mixture had the following composition: 50 mM glycyl-glycine, 10 mM sodium arsenate, 3.2 mM dithiothreitol, 0.25 mM NAD^+, 1.2 units/ml of GAPDH, 2.5 mM $CaCl_2$, 0.08 mM ThDP, and 2.5 mg/ml of phosphopentose mixture, pH 7.6. The reaction was started by addition of TK.

Titration of the active centers of TK was carried out during the incubation of 0.5 mg/ml apo-TK in 50 mM glycyl-glycine buffer, pH 7.6, with equimolar and lower concentration of ThDP in the presence of calcium, followed by the measurement of the activity of the formed holoenzyme, which corresponded to the quantity of the bound ThDP [10].

RNA concentration was measured in the reaction with orcine. Yeast RNA was used as a standard [11].

Chromatography on Sephacryl S-300 was carried out on the column (1.25 × 31 cm) equilibrated with 50 mM potassium phosphate buffer, pH 7.6. The same buffer was used for elution. The elution velocity was 0.5 ml/min with fraction volume 1 ml. The volume of applied enzyme solution was 1 ml. β-Amylase (200 kD), aldolase (160 kD), free TK (148.4 kD), and lactate dehydrogenase (140 kD) were used as markers.

CD spectra were recorded on Jobin Ivon Mark V dichrograph (France) modified and conducted by an IBM-PC1 computer in a cuvette with optical pathlength 1 cm. The data were processed with the use of the RDA program.

RESULTS AND DISCUSSION

Identification and properties of TK–RNA complex. It was demonstrated by Tikhomirova and Kochetov [1] that during TK isolation by an immunoaffinity method an enzyme with higher molecular weight and lower specific activity in comparison to the free enzyme was eluted from the immunoaffinity column. The maximum in the absorption spectrum for this enzyme was shifted to shorter wavelengths (the spectra were taken after chromatography on Sephacryl S-300). After rechromatography of this sample on Sephacryl S-300 the volume of the protein yield increased to the level typical for the free enzyme, the specific activity increased, and the absorption spectrum of the free enzyme was restored. In addition, a non-protein component was eluted from the column with maximal absorption at 260 nm. Incubation of the high molecular weight preparation with RNase A led to an increase in its catalytic activity 20-fold. It was concluded that the previously unknown TK form represented a complex of the enzyme with RNA. This was confirmed by a positive reaction to pentoses and with ethidium bromide, and also by the hydrolysis of the free non-protein component to bases [1].

During TK elution from freshly dried yeast only an enzyme with molecular weight 210 kD (TK-210) was eluted from the column. The molecular weight was determined using Sephacryl S-300. The specific activity of the enzyme was 0.8-1 unit/mg. On storage of this enzyme frozen in 50 mM potassium phosphate buffer, pH 7.6, for 7 days or more its molecular weight was decreased to 160 kD (TK-160), and the specific activity increased to

3.5-6 units/mg. A profile of elution from the column with Sephacryl S-300 of one of the preparations of TK is presented in Fig. 1. This preparation was extracted with ammonia (in conditions when the high molecular weight complex was partly converted to the low molecular weight complex, see below). The molecular weight of TK in the first peak was 210 kD, specific activity was 0.8 unit/mg; the molecular weight of TK in the second peak was 160 kD, specific activity 6.3 units/mg.

The assessment of the number of nucleotides in RNA bound to TK by ribose content showed that 180 nucleotides were bound to TK-210 and 20 nucleotides to TK-160 (the calculation was carried out considering the mean molecular weight of nucleotide being 321 daltons). These results correlated with data on the measurement of the molecular weight of TK preparations on Sephacryl S-300. The length and the number of RNA chains were not defined. Ribose was not detected in the free (not bound to RNA) TK with molecular weight 148.4 kD [12]. The specific activity of the free TK was 20 units/mg. Thus, the TK–RNA complex had molecular weight of 210 kD, and its specific activity represented 4-5% of the activity of the free enzyme. During storage of the complex its molecular weight decreased to 160 kD, and the specific and total activities of the enzyme increased to 15-30% of the activity of the free enzyme, i.e., the high molecular weight complex was reduced to the low molecular weight complex. A complex with an intermediate molecular weight between 210 and 160 kD was never detected.

The titration of the active centers of TK-160 demonstrated that during incubation of the equimolar concentrations of apoTK and ThDP 50% of the enzymatic activity was detected when measured without addition of the cofactors, i.e., one ThDP molecule was bound to one dimeric molecule of the enzyme. Consequently, all TK-160 molecules bound ThDP as well as the molecules of the free TK.

The complex was identified by its absorption spectrum [1]. The data are presented in Fig. 2. The complex TK-210 had the maximum of absorption at 260 nm in comparison to 280 nm for the free TK (curves *1* and *3*, respectively, in Fig. 2). The absorption maximum for the TK-160 complex was shifted to 265 nm with a larger peak (curve *2* in Fig. 2). For the free TK the ratio of the absorption at 280/260 nm wavelengths was 1.86-1.95, for TK-160 0.78-0.88, and for TK-210 0.63-0.67. The data are presented for enzyme preparations that were run through Sephacryl S-300 equilibrated with 50 mM potassium phosphate buffer, pH 7.6.

We recorded the CD spectra of the three enzyme forms (Fig. 3). In comparison to the free TK, for the apo- and holoforms of TK-160 and TK-210 the maximum of

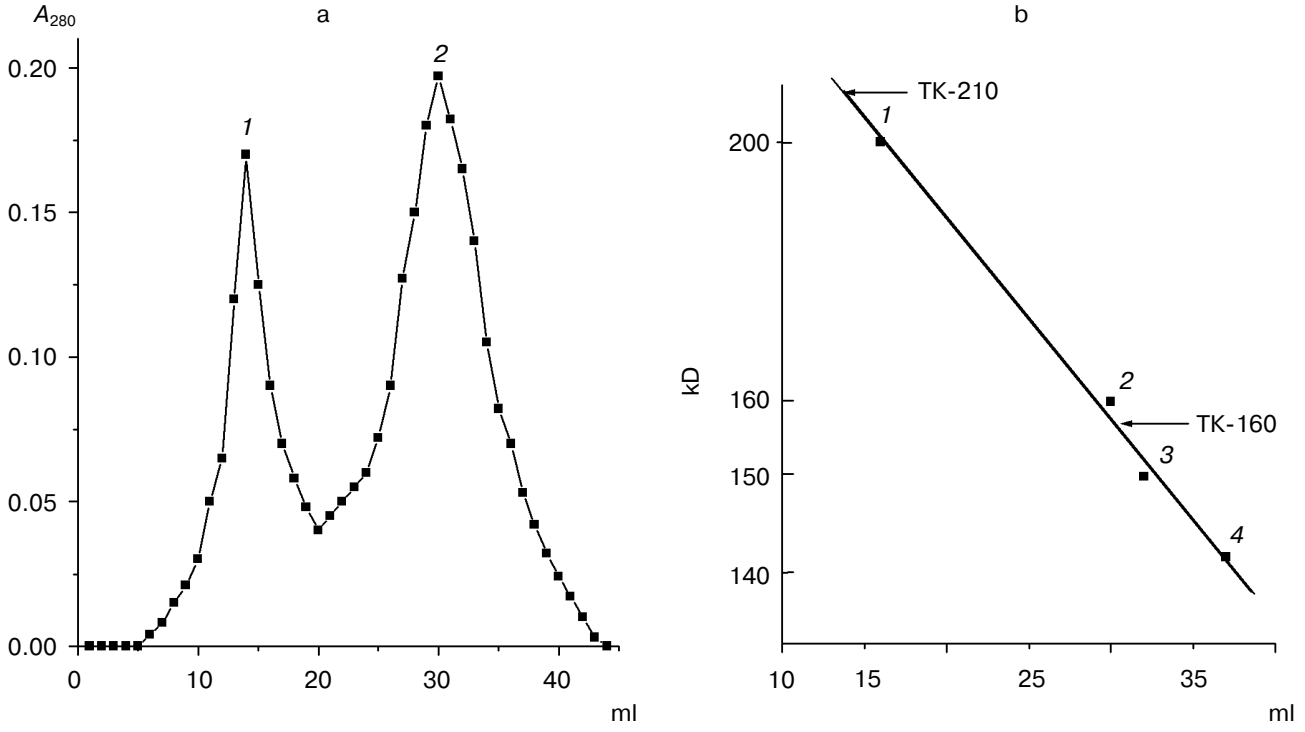

Fig. 1. a) Chromatography of TK on Sephacryl S-300 in 50 mM potassium phosphate buffer, pH 7.6: TK-210 (*1*), TK-160 (*2*); b) calibration curve for the assessment of the molecular weight of TK. Marker proteins: β-amylase (*1*, 200 kD), aldolase (*2*, 160 kD); free TK (*3*, 148.4 kD), lactate dehydrogenase (*4*, 140 kD).

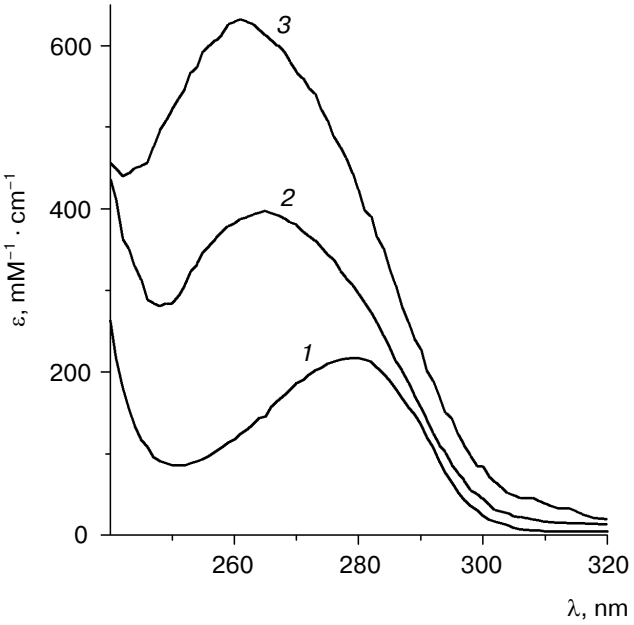

Fig. 2. Absorption spectra of TK: free (*1*), TK-160 (*2*), TK-210 (*3*). All spectra were taken in 50 mM potassium phosphate buffer, pH 7.6.

the positive absorption band was shifted from 285 to 266 nm (curves *1* and *2* in Fig. 3, a, b, and c). The magnitude of Δε of the positive maximum for the apoenzyme was 0.95 for the free TK, 2.3 for TK-160, and 8.1 for TK-210. We can indicate for comparison that for the free RNA only the positive band with maximum at 265 nm, $\Delta\varepsilon = 4.8$ was observed [13]. A negative absorption band appeared for the holoform of the free TK with maximum at 320 nm (curve *2* in Fig. 3a). The magnitude of the peak of the negative absorption band for the holoform of TK-160 was significantly decreased in comparison to the free TK (curve *2* in Fig. 3, a and b), while the negative absorption band was practically absent for the holoform of TK-210 (curve *2* of Fig. 3c).

After the addition of hydroxypyruvate (donor substrate) to the holoenzyme the inversion of the 320 nm band occurred only for the free TK (curve *3* in Fig. 3a). For TK-160 the negative absorption band disappeared, but inversion was not observed (curve *3* in Fig. 3b). After the following addition of glycolaldehyde (acceptor substrate) the negative absorption band was restored for the free TK and TK-160 (curve *4* in Fig. 3, a and b). Simultaneously the molar coefficient of dichroic absorption at 275 nm increased from 0.475 to 10 for the free TK (curve *4*, Fig. 2a), which corresponds to the formation of 31 M erythrulose (extinction coefficient $50 \cdot 10^{-5}$ at 275 nm for 1 mM erythrulose [9]), and from 7.7 to 13.3 for the TK-160, which corresponds to the formation of 11 M erythrulose (curve *4*, Fig. 3b). This comprises 35% of the product formed in the reaction with free TK, which cor-

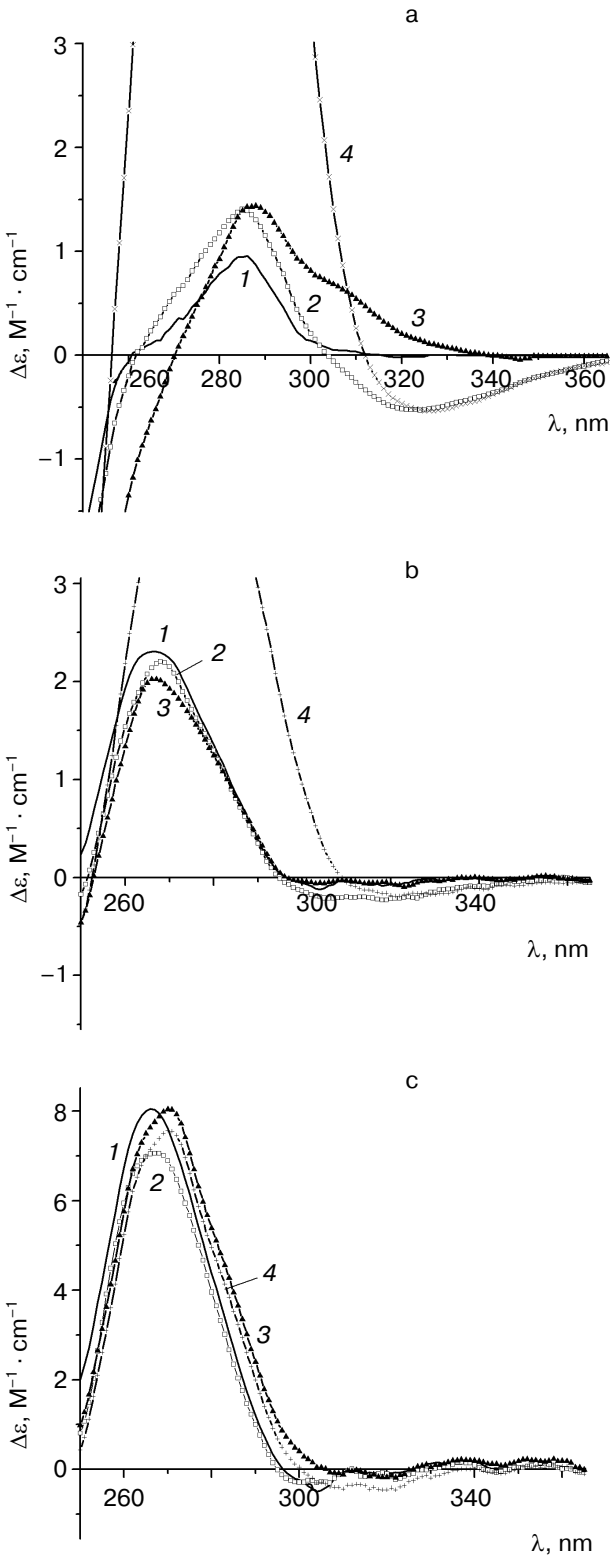

Fig. 3. CD spectra of TK (free (a); TK-160 (b), and TK-210 (c)) in near UV (all spectra were taken in 50 mM glycyl-glycine buffer, pH 7.6, containing 2.5 mM $CaCl_2$): apoTK (*1*), holoTK (*2*), holoTK with addition of 2 mM hydroxypyruvate (*3*), the same as (*3*) with addition of 10 mM glycolaldehyde (*4*).

responds to the difference in the specific activities of these two forms of the enzyme. No changes in the area of 275 nm were observed for TK-210 (curves *3* and *4* in Fig. 3c).

The affinities to ThDP in the presence of Ca^{2+} and to X5P (donor substrate) were not changed for the free TK and TK-160: K_m for ThDP was $(3.2\text{-}3.6)\cdot10^{-8}$ M and $(4.7\text{-}5.0)\cdot10^{-7}$ M (for the first and the second active centers of TK, respectively); K_m for X5P was $(5.8\text{-}7.7)\cdot10^{-5}$ M. However, the time required for the binding of calcium changed: for the complete expression of TK-160 and TK-210 activities it was necessary to incubate the enzyme with calcium for not less than 1 min before the addition of ThDP and substrates, or the enzyme with calcium and ThDP before the addition of substrates. Otherwise, the enzymatic activity represented only 5% of the maximal possible activity. This effect is not yet understood.

Conditions of dissociation of the TK–RNA complex. The high molecular weight complex of TK with RNA (TK-210) was relatively labile. It dissociated spontaneously to the low molecular weight complex (TK-160) during storage of both dry yeast and frozen enzyme preparations. TK forms were identified by measurement of the molecular weight on Sephacryl S-300 followed by recording of absorption and CD spectra and by the measurement of the specific activity. The increase of the enzymatic activity during preincubation of the enzyme with calcium before the addition of the substrate was considered as a reliable criterion of the presence of the complex. We managed to isolate TK-210 from dry yeasts only when the yeast was stored for less than 7 days. After the storage of yeast for more than 7 days, but less than 2 months, we isolated a mixture of TK-160 with the free TK. For isolation of the free TK, dry yeast was stored for not less than 2 months.

The low molecular weight complex of TK with RNA (TK-160) was stable for at least 6 months during storage of the enzyme in 50% saturated ammonium sulfate solution, pH 7.6. For dissociation of TK-160 to RNA and the free enzyme, the complex was incubated in ammonium sulfate of 40% saturation, pH 7.6-8.0, at protein concentration 1 mg/ml for 24-48 h at 4°C. If dissociation was incomplete, TK-160 and the free TK were fractionated in ammonium sulfate: the undissociated complex was separated by centrifugation at up to 40% saturation with ammonium sulfate, while the free TK was precipitated in the range of ammonium sulfate concentrations 55-65%. The release of RNA from the enzyme occurred under the same conditions in the yeast extract during its incubation in the solution of ammonium sulfate before loading on the immunoaffinity column (ammonium sulfate was removed before loading by dialysis). In the latter case the degree of saturation with ammonium sulfate varied from 20 to 40% depending on the conditions of solubility of the complex, since dissociation occurred only in solution, but accelerated with increase in ammonium sulfate concentration.

The specific activity of the low molecular weight complex after RNA release by ammonium sulfate increased from 6 to 12 units/mg, although the specific activity of the free TK isolated from the same batch of yeast was 20 units/mg. This was apparently due to partial inactivation of the enzyme during long isolation procedures.

The enzyme was isolated in the form of a complex with RNA from yeast that were grown from June to November, the selection of yeasts was carried out both during the growth and stationary (1 h before reseeding) phases. Only free TK was isolated from yeast grown from December to April.

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