THE PURIFICATION AND SOME PROPERTIES OF BREWER'S YEAST APOTRANSKETOLASE

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Received 26 December 1973

1. Introduction

Transketolase (Sedoheptulose-7-phosphate: D-Glyceraldehyde-3-phosphate glycolaldehydetransferase, EC 2.2.1.1) has been isolated and purified from Baker's yeast [1,2], candida yeast [3], spinach [4,5], rat and pork liver [6,7], and human erythrocytes [8].

Recently, Kochetov, et al. [9] reported that transketolase from Baker's yeast contained glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) as a 'functionally-bound' enzyme. The similarity of the physicochemical properties of both enzymes, especially of their molecular weight, has made it difficult to separate them [8] although purification was successfully made with CM-sephadex C-50 [9]. We have found that transketolase could be obtained free from glyceraldehyde-3-phosphate dehydrogenase very conveniently with Sephadex G-200 if the enzymes were previously exposed to glycylglycine buffer of pH 7.6 at 0°C for 48 hr. The dehydrogenase seemed to dissociate into its subunits under this experimental condition and hence it was eluted later than transketolase by gel filtration. Several properties of apotransketolase including the inhibition of its reconstitution by ethylenediamine tetraacetate (EDTA) or phenylglyoxal are presented in order to obtain a better understanding of the nature of the binding of both metal ion and thiamine pyrophosphate (TPP) to apotransketolase.

2. Materials and methods

Ribose-5-phosphate barium salt, NADH and TPP were purchased from Seikagaku Ind., Oriental Yeast Ind. and Tokyo Kasei respectively. A mixture of α-glycerophosphate dehydrogenase and triose phosphate isomerase was prepared from rabbit muscle [2]. The pentose-5-phosphate equilibrium mixture was prepared according to De la Haba, et al. [1]. Transketolase was isolated from 'Sapporo' brewer's yeast and was purified according to the procedure of Srere, et al. [2] with the following modifications. The eluate of 0.005 M phosphate buffer (pH 7.7) from DEAE-cellulose column was fractionated with ammonium sulfate. The 59–65% saturated ammonium sulfate precipitate was dissolved in the same buffer and was rechromatographed on a DEAE-cellulose column (2.5 × 20 cm) with 0.005 M phosphate buffer (pH 7.7) at a flow rate of 30 ml/hr. The fractions of 80–120 ml were collected and fractionated again with ammonium sulfate to obtain 59–65% precipitate which was dissolved in glycylglycine buffer (pH 7.7) to make a final buffer concentration of 0.025 M. The solution was kept at 0°C for 48 hr for the complete resolution of cofactors [10]. The precipitate from 50–70% ammonium sulfate (pH 7.6) saturation was dissolved in 0.005 M phosphate buffer (pH 7.7) and applied to a Sephadex G-200 column (1.5 × 95 cm) preequilibrated with 0.005 M phosphate buffer (pH 7.7) for 72 hr. The fraction of 83–94 ml was collected and rechromatographed with Sephadex G-200. Electrophoresis of the enzyme was done in 7.5% polyacrylamide gels (pH 8.9) as described by Davis [11]. Transketolase activity was determined spectrophotometrically by the method of coupling the transketolase with triose isomerase and α-glycerophosphate dehydrogenase-NADH [1]. The reaction mixture consited of 100 μmol of glycylglycine buffer (pH 7.6), 9 μmol of $MgCl_2$ 100 nmol of TPP, 300 nmol of NADH, 1.0 mg of α-glycerophos-

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phate dehydrogenase-triose phosphate isomerase, 200 μg of transketolase, and 3.0 μmol of pentose 5-phosphate equilibrium mixture. The reaction was initiated by adding pentose equilibrium mixture to a total volume of 3.0 ml and the absorbancy decrease of NADH was measured continuously for a few minutes before and after the start of the reaction at 30°C using Hitachi–Perkin–Elmer spectrophotometer (Model 139) with the recording attachment (Model QPD54). The activity of glyceraldehyde phosphate dehydrogenase was determined by the method of Oguchi [12].

A modified Dische's method [13] was used for the colorimetric determination of sedoheptulose, one of the products of transketolase reaction, in the following inhibition experiments with EDTA or phenyl glyoxal. Apotransketolase (300 μg) was incubated with 5 nmoles–50 μmoles of EDTA or 15–100 μmoles of phenylglyoxal in phosphate buffer (pH 8.0). After the addition of TPP and divalent metal ion, the reaction mixture was again incubated and filtered with Sephadex G-25. An aliquot of the eluents was used for checking the enzyme activity. Apoenzyme which was incubated with a TPP and metal ion mixture followed by the addition of an inhibitor for the same period of time were also gel-filtered and their enzyme activities were examined.

The determination of the molecular weight of transketolase was performed by gel filtration with Sephadex G-200 as described by Andrews [14] and by SDS-polyacrylamide disc electrophoresis by the method of Dunker [15]. The following proteins in the calibration kit of pharmacia were used with Sephadex G-200 for the calibration curve: rabbit muscle aldolase (mol. wt. = 147 000), bovine serum albumin (67 000), ovalbumin (45 000), bovine pancreas chymotrypsinogen A (25 000) and sperm whale myoglobin (17 800). A Sephadex G-200 column (1.5 × 95 cm) which had previously been equilibrated with 0.005 M phosphate buffer (pH 7.7) was used for applying 7 mg of apotransketolase. The same buffer was used for the upward elution at the flow rate of 8.0 ml/hr. The disc electrophoresis was carried out with 5% polyacrylamide gels (pH 7.6), using bovine serum albumin (mol. wt. = 67 000), bovine liver catalase (60 000 as a monomer), ovalbumin (45 000) and bovine pancreas chymotrypsinogen (25 000) as the marker proteins. Samples were incubated in 0.01 M phosphate buffer (pH 7.2) containing 25% glycerol, 1% SDS, and 3% 2-mercaptoethanol at 25°C overnight after which 10 μl (3 μg of protein) was placed on to the gel. The gel was run at 8 mA per tube at room temperature and was stained with 0.5% amido black 10B–20% acetic acid for detecting the protein band and electrophoretically destained with 7% acetic acid. Protein was determined by the method of Lowry, et al. [16].

3. Results

Table 1 summarizes the results of the purification of apotransketolase. The details of the procedure were described in Materials and methods. The specific activity obtained at step 7 represents a 1340-fold purification. Before applying the sample to Sephadex G-200, it was kept at 0°C for 48 hr for the removal of cofactors from the enzyme. The pH was quite critical for resolution. Transketolase activities of 65% and 22% were found upon treatment at pH 6.8 and 7.2, respectively; whereas, complete resolution was accomplished at pH 7.6, 8.0, or 8.5. The elution profile of apotransketolase through Sephadex G-200 is presented in fig. 1. The results of gel electrophoresis show that fractions 83–94 ml contained transketolase activity only, that fractions 106–118 ml contained both transketolase and glyceraldehyde-3-phosphate dehydrogenase activity and that fractions 130–140 ml contained the latter enzyme activity only.

The determination of the molecular weight of apotransketolase using gel filtration was made from the calibration curve obtained with several protein

Table 1
Summary of purification of brewer's yeast transketolase.

Fraction	Specific activity ($\Delta_{340\ nm} \times 10^3$/min/mg protein	Purification
1. Crude extract	9	(1)
2. Acetone fraction	18	(2)
3. Heat treatment	47	(5)
4. First DEAE-cellulose	110	(12)
5. $(NH_4)_2SO_4$	226	(25)
6. Second DEAE-cellulose	1660	(184)
7. Sephadex G-200	12060	(1340)

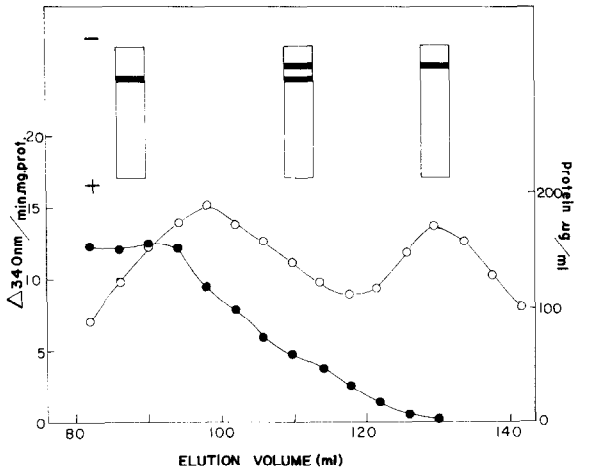

Fig. 1. Chromatography of the apoenzyme on Sephadex G-200. Elution and assay procedures are described in the text. Fractions of 83–94 ml were pooled for further purification by Sephadex G-200. The results with polyacrylamide gel electrophoresis for the 3 fractions of the Sephadex G-200 column are shown in the upper part of the figure. The gel containing 7.5% acrylamide (pH 8.3) was run at 2–3 mA per tube at room temperature and was stained for protein with 0.2% Amido black 10B in methanol–water–acetic acid (5:5:1) and electrophoretically destained with 7% acetic acid. (o—o—o) Protein; (•—•—•) transketolase activity.

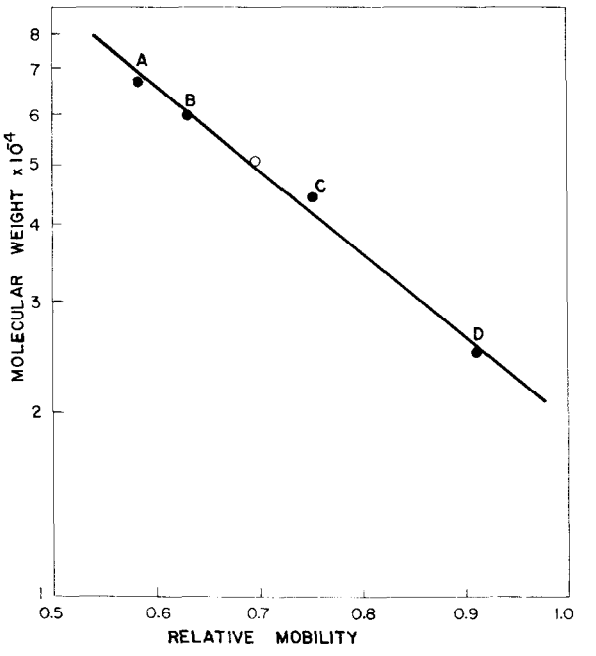

Fig. 2. Plot of log molecular weight against distance of migration on the SDS polyacrylamide gel. A) bovine serum albumin, 67 000; B) bovine liver catalase, 60 000; C) ovalbumin, 45 000; D) bovine pancreas chymotrypsinogen A;(O) apotransketolase.

standards [14]. From elution fraction 93 ml, it was calculated that the molecular weight of apotransketolase is approximately 100 000 ± 6000. Fig. 2 represents the results of SDS-polyacrylamide disc electrophoresis. A linear relationship was obtained between the molecular weight of the proteins and their mobilities. Bovine liver catalase is assumed to be dissociated into 4 subunits giving a monomer molecular weight of 60 000 [17]. The given data are the mean values of four repeated experiments. The molecular weight of apotransketolase was calculated to be 50 000 ± 2500–3000. It is understandable that apotransketolase can be dissociated into 2 subunits under these experimental conditions since the enzyme was exposed overnight to 2-mercaptoethanol at 25°C.

The effect of EDTA on the reconstitution of apotransketolase is shown in fig. 3. A complete inhibition was observed when apotransketolase was incubated with 0.5–5 μmoles of EDTA followed by the addition of 1 μmole of TPP. The inhibition by EDTA, however, was much smaller when apotransketolase was preincubated with cofactors as observed in the reconstitution study of pyruvate decarboxylase [18]. The inhibition of apotransketolase by EDTA was reversible. The addition of 0.5 μmole of Ca^{2+}, Mn^{2+} or Mg^{2+} to transketolase whose activity was inhited by 0.25 μmole of EDTA, restored the full enzyme activity in the presence of 0.1 μmole of TPP. The activation of apotransketolase by several divalent metals showed the following order with 1 nmole of TPP; $Mn^{2+} > Ca^{2+} > Mg^{2+}$ at 3×10^{-4} M – 3×10^{-6} M, $Ca^{2+} > Mn^{2+} > Mg^{2+}$ at 3×10^{-3} M concentration [19].

The inhibitory effects of phenylglyoxal on the reconstitution of transketolase is presented in table 2. The previous addition of phenylglyoxal to the apoenzyme inhibited the reconstitution of apotransketolase very strongly, although the activity of holoenzyme was also inhibited slightly by the reagent. The interaction between the phosphate group of TPP and the guanido residue of arginine at the coenzyme binding center of apoenzyme may be suggested since phenyl-

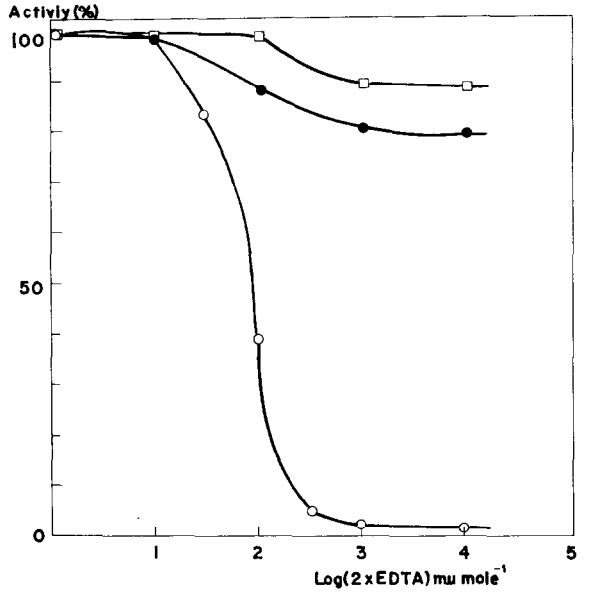

Fig. 3. Inhibitory effect of EDTA on the reconstitution of transketolase. Apotransketolase (300 μg) was incubated with 100 nmoles of TPP (□——□——□) or with 10 nmoles of TPP and 0.1 μmole of $CaCl_2$ (●——●——●) for 30 min at 30°C. EDTA was then added for incubation for 10 min at 30°C. Apoenzyme was also incubated with EDTA for 10 min at 30° followed by the additional incubation with 1 μmole of TPP for 30 min at 30°C (○——○——○). Transketolase activity was determined colorimetrically as described in the text.

Table 2
Inhibitory effect of phenylglyoxal on the reconstitution of transketolase.

	Phenylglyoxal		
Activity	15 μmole	75 μmole	100 μmole
(A)	17	15	6
(B)	10	1	0

For (A), 300 μg of apotransketolase was incubated with 4.5 μmoles of $MgCl_2$ and 100 nmole of TPP in 0.02 M phosphate buffer (pH 8.0) in a total volume of 0.5 ml for 30 min at 30°C. Phenylglyoxal was then added for incubation for 10 min at 30°C. The incubated mixture was filtered through Sephadex G-25 (10 × 350 mm) which had previously been equilibrated with 0.005 M phosphate buffer (pH 7.6), and an aliquot was used for determining the transketolase activity colorimetrically [13]. For (B), the same amounts of apoenzyme and $MgCl_2$ as used for (A) were incubated in 0.02 M phosphate buffer with phenylglyoxal for 10 min at 30°C followed by the addition of 100 nmole of TPP for further incubation at 30°C for 30 min. The incubated mixture was treated as described above. Apoenzyme which was incubated with $MgCl_2$ only for 10 min before adding 100 nmole of TPP and treated in the same way as described above was used as a reference. The amount of sedoheptulose formed in the reference was 24.0 μg/hour/mg of protein.

glyoxal is known to be a highly specific reagent for arginine in the short period of time required for the reaction. [20].

4. Discussion

Kochetov et al. [9] have demonstrated that purified transketolase from Baker's yeast contains glyceraldehyde phosphate dehydrogenase as a functionally bound enzyme. The former enzyme was successfully separated by employing CM-Sephadex C-50 column, although the complex of both enzymes was still observed in the first eluent. Our results with Sephadex G-200 showed that the two enzymes could be clearly separated upon the exposure of the enzyme complex to glycylglycine buffer of pH 7.6 for 48 hr at 0°C. This treatment gave not only the complete resolution of transketolase from TPP and metal ions but also the dissociation of glyceraldehyde phosphate dehydrogenase (mol. wt. = 140 000) into its subunits. This would explain why the latter enzyme was eluted later than the former in Sephadex G-200 filtration. The molecular weight of apotransketolase was estimated to be approximately 100 000 in gel filtration and 50 000 in SDS-polyacrylamide disc electrophoresis. Molecular weights of 100 000 for spinach transketolase [5], and 140 000 for baker's yeast transketolase [21,22] are reported. It is known that brewer's yeast pyruvate decarboxylase (mol. wt. = 209 000) is dissociated into subunits of one-half the molecular weight of the native enzyme in Tris buffer (pH 7.5, 8.0) and that the subunits form a stable dimer in the presence of TPP and Mg^{2+} at pH 6.5 [23]. Therefore, it may be possible that holotransketolase has a molecular weight twice that of apotransketolase.

The interaction of TPP and divalent cations with apoenzymes has been of recent interest for several investigators [18,24–27]. Schellenberger, et al. [24]

have suggested 2 independent binding sites on apopyruvate decarboxylase, one for TPP and another one for metal. They also postulated the formation of a ternary complex in which TPP is tightly bound to apoenzyme through its 1′-*N* of pyrimidine molecule and thiazole pyrophosphate group. This assumption seems to have indirect support from the fact that 4-methyl-5-(pyrophosphonyl-2-hydroxyethyl) thiazole could not bind with apoenzyme tightly and was separated by gel filtration [18]. Our preliminary results [28] also showed that 3-methyl or 3-benzyl thiazole pyrophosphate analogues were eluted separately from apopyruvate decarboxylase through Sephadex G-25, suggesting the importance of 'pyrimidine' for tighter binding. The inhibitory effect of EDTA on enzyme reconstitution is probably based on the chelation with the metal still present in apotransketolase, protecting thiamine pyrophosphate from 'cyclic' binding. Thus the preincubation of apotransketolase with TPP (0.1 μmole) alone gave slightly higher protection than that with both TPP (0.01 μmole) and $CaCl_2$ (0.09 μmole). While the inhibition of EDTA (0.25 μmole) was reversible, that of phenylglyoxal was irreversible because of the probable covalent bonding with the guanido group. It is interesting that TPP could protect the inhibition by phenylglyoxal, though the interaction between the pyrophosphate group of TPP and the apoenzyme is presumably one of ionic nature.

Acknowledgements

We would like to exprss our gratitude to Dr. C. Kawasaki of Koube Gakuin University for his interest and continuous encouragement.

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