CRYSTALLINE THREONINE ALDOLASE FROM *CANDIDA HUMICOLA*

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Received February 10, 1970

Summary.

The preparation of crystalline threonine aldolase from the cell extract of *Candida humicola* is described. The enzyme is homogeneous upon ultracentrifugation and disc electrophoresis. The molecular weight is 277,000. The enzyme exhibits an absorption maximum at 420 mμ, and contains 6 moles of pyridoxal phosphate per mole of enzyme. The enzyme catalyzes the stoichiometric conversion of *L*-threonine into acetaldehyde and glycine. The enzyme also catalyzes the glycine formation from *D,L*-allothreonine and *L*-serine.

Threonine aldolase (*L*-threonine acetaldehyde-lyase, EC 4.1.2.5) has been shown to catalyze the stoichiometric conversion of *L*-threonine into acetaldehyde and glycine (equation I) and require pyridoxal phosphate as

L-Threonine ⇌ acetaldehyde + glycine (I)

a cofactor. The enzyme was found in the liver and kidney of certain animals (Braunstein *et al.*, 1949; Lin *et al.*, 1954; Karasek *et al.*, 1957; Berkersky *et al.*, 1968). The occurrence of this enzyme activity in microorganisms has recently been investigated in our laboratory. We found that a strain of *Candida humicola* produced a markedly high activity of the enzyme. This communication describes the preparation of crystalline threonine aldolase from the cell extract of *C. humicola*, and some of its properties.

Purification and Crystallization.

C. humicola (IFO 0760) was grown in 40 liters of a medium containing 0.2% *L*-threonine, 0.2% KH_2PO_4, 0.1% $(NH_4)_2SO_4$, 0.1% $MgSO_4$ and 0.05% yeast extract, with aeration, for 14 hours at 30°. The cells were

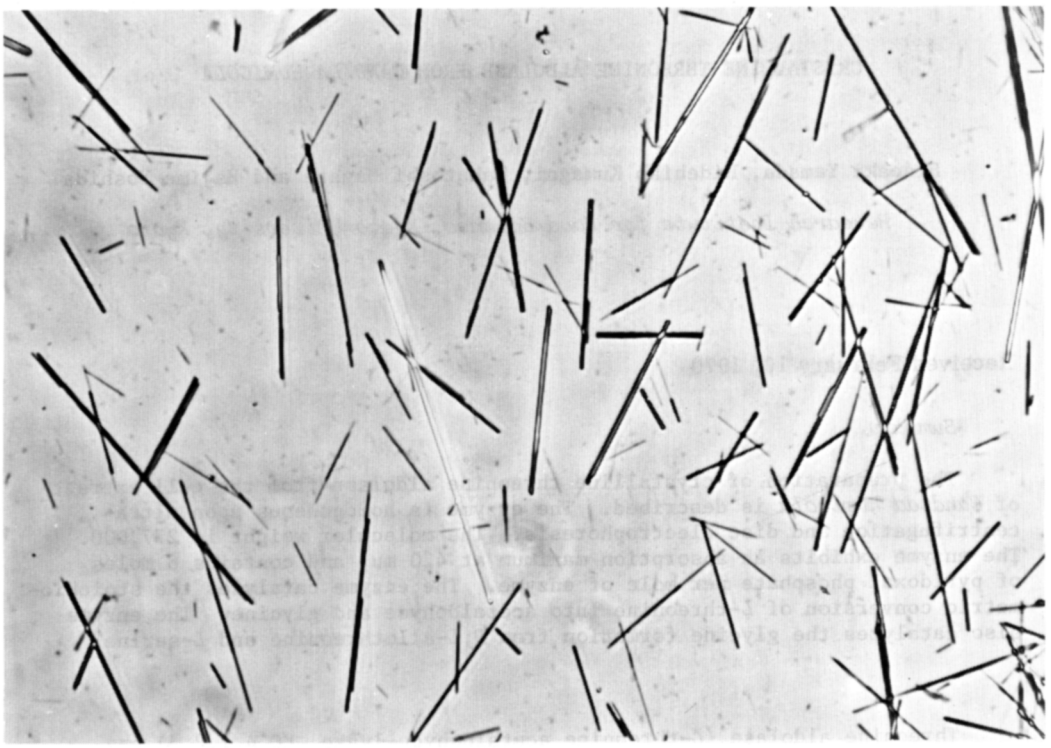

Fig. 1. Photomicrograph of crystalline threonine aldolase (x 600).

harvested, washed and suspended in 0.03 M potassium phosphate buffer, pH 6.4. All subsequent procedures were performed at 0-5°. *Step I.* The cell suspension was subjected to ultrasonic oscillation (20 Kc, for 5 hours) and centrifuged. *Step II.* The supernatant solution was applied to a DEAE-Sephadex column (6 x 70 cm) equilibrated with 0.03 M potassium phosphate buffer, pH 6.4. After the column was washed with 4 liters of 0.1 M potassium phosphate buffer, pH 6.4, containing 0.1 M KCl, the enzyme was eluted with 0.1 M potassium phosphate buffer, pH 6.4, containing 0.4 M KCl, 5×10^{-3} M mercaptoethanol and 1×10^{-3} M EDTA. The active fractions were combined and concentrated by the addition of ammonium sulfate (50% saturation). The precipitate was collected and dialyzed against a dialysis buffer which contained 0.03 M potassium phosphate, pH 6.4, 5×10^{-3}

Table I. Purification of threonine aldolase from *C. humicola*

Threonine aldolase was assayed in a reaction mixture containing enzyme, 50 μmoles of *L*-threonine, 0.2 μmoles of pyridoxal phosphate, 400 μmoles of KCl and 400 μmoles of Tris-chloride buffer, pH 8.6, in a total volume of 3.5 ml. The reaction was carried out at 30° for 15 minutes and stopped by the addition of 1 ml of 30% trichloroacetic acid. The amount of acetaldehyde formed was determined with the deproteinized filtrate by a modification (Soda, 1967) of the method of Paz *et al.* (1965). A unit was defined as the amount of enzyme which catalyzed the formation of 1 μmole of acetaldehyde per minute under the assay conditions. The protein concentration was determined spectrophotometrically by measuring the absorbancy at 280 mμ. An *E* value of 3.98 for 10 mg per ml and for 1-cm light path which was obtained by absorbancy and dry weight determinations, was used throughout.

Step	Fraction	Total protein	Total units	Specific activity
I	Crude extract	103,300	593	0.00573
II	DEAE-Sephadex	1,170	170	0.145
III	Hydroxylapatite	109	156	1.43
IV	Sephadex G-200	58.8	150	2.56
V	Crystals I	47.5	130	2.75
	Crystals II	30.9	90	2.91

M mercaptoethanol, 1 x 10^{-3} M EDTA and 1 x 10^{-5} M pyridoxal phosphate. *Step III.* The dialyzate was applied to a hydroxylapatite column (3 x 5 cm) equilibrated with the dialysis buffer. After the column was washed with 350 ml of the dialysis buffer, the enzyme was eluted with 0.1 M potassium phosphate buffer, pH 6.4, containing 5 x 10^{-3} M mercaptoethanol, 1 x 10^{-3} M EDTA and 1 x 10^{-5} M pyridoxal phosphate. The active fractions were combined and concentrated with ammonium sulfate (50% saturation). The precipitate was collected and dissolved in a minimum amount of the dialysis buffer. *Step IV.* The enzyme solution was passed through a Sephadex G-200 column (2 x 100 cm) equilibrated with the dialysis buffer. The active fractions containing enzyme of specific activity greater than 2 were combined and concentrated with ammonium sulfate (50% saturation). The precipitate was collected and dissolved in a minimum amount of the dialysis buffer. *Step V.*

Finely powdered ammonium sulfate was cautiously added to the enzyme solution until it became slightly turbid, and the mixture was placed in an ice bath. Crystallization began after about 2 hours and virtually complete within 3 days. Fig. 1 is a photomicrograph of the crystalline threonine aldolase of *C. humicola*, which appears as fine needles with a yellow color. Recrystallization was carried out by repeating the last step. A summary of typical purification procedure is presented in Table I.

Properties.

The crystalline threonine aldolase preparation gave a single band on acrylamide gel electrophoresis carried out in Tris-glycine buffer, pH 8.3, (Davis, 1964) containing 1×10^{-2} M KCl, 5×10^{-3} M mercaptoethanol and 1×10^{-5} M pyridoxal phosphate. The enzyme preparation sedimented as a single symmetric peak under ultracentrifugation performed at pH 6.4 in the dialysis buffer. Extrapolation of the data obtained from four ultracentrifuge runs to zero protein concentration gave an $S^0_{20,w}$ of 11.6×10^{-13} (cm/sec). A diffusion constant, $D_{20,w}$, of 4.06×10^{-7} (cm^2/sec) was determined for a 6.40 mg/ml solution of protein. A value of 277,000 was calculated for the molecular weight of the enzyme assuming a partial specific volume of 0.75. The absorption spectrum of the enzyme has a maximum at 420 mμ (Fig. 2), indicating that the formyl group of the bound pyridoxal phosphate forms an azomethine link to an amino group of the protein, as in other pyridoxal phosphate enzymes. The pyridoxal phosphate content of the enzyme determined by the equilibrium dialysis technique (Newton *et al.*, 1965) was 1 mole of pyridoxal phosphate per 46,200 g of protein, implying 6 moles of pyridoxal phosphate per mole of enzyme. Dialysis of the enzyme against 0.03 M potassium phosphate buffer, pH 6.4, containing 1×10^{-1} M cysteine, 5×10^{-3} M mercaptoethanol and 1×10^{-3} M EDTA led to a complete resolution of the enzyme from pyridoxal phosphate. In the absence of pyridoxal phosphate, the resolved enzyme has less than 5% of the activity

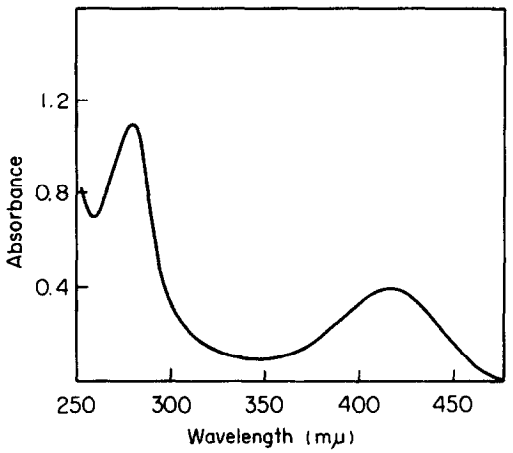

Fig. 2. Absorption spectrum of crystalline threonine aldolase (2.76 mg per ml) in 0.03 M potassium phosphate buffer, pH 6.4, containing 5 x 10^{-3} M mercaptoethanol and 1 x 10^{-3} M EDTA.

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of the original holoenzyme and retains more than 95% of its activity in the presence of excess pyridoxal phosphate. The concentration of pyridoxal phosphate to give the half maximum rate of the reaction (equation I) with the resolved enzyme was determined to be 2.5 x 10^{-7} M.

The crystalline threonine aldolase catalyzes the stoichiometric conversion of *L*-threonine and *D,L*-allothreonine into acetaldehyde and glycine. This conversion is apparently reversible to a slight extent, although the configuration of the product formed in the reverse reaction has not been established. The enzyme also catalyzes the reversible conversion of *L*-serine into formaldehyde and glycine (equation II).

L-Serine $\rightleftharpoons$ formaldehyde + glycine (II)

Tetrahydrofolate which is known to be a cofactor of the serine trans-hydroxymethylase-catalyzed reaction in animal liver (Schirch *et al.*, 1962), is rather inhibitory for the interconversion of *L*-serine and glycine by the threonine aldolase of *C. humicola*. The concentration of these substrates to give the half maximum rate of the glycine formation was found to be 5.5 x 10^{-4} M, 3.9 x 10^{-4} M and 3.7 x 10^{-3} M for *L*-threonine, *D,L*-allothreonine

and *L*-serine, respectively. The optimal pH was 8.5 for *L*-threonine and *D,L*-allothreonine, and 10.0 for *L*-serine, in Tris-chloride buffer.

Acknowledgments.

We wish to thank Emeritus Prof. H. Katagiri, Prof. K. Ogata, Prof. Y. Morita and Prof. T. Tochikura, Kyoto University, Kyoto, for their interest and advice during the course of this work.

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