ALLOSTERIC BEHAVIOR OF YEAST THREONINE DEAMINASE UNDER PARTIALLY INACTIVATING CONDITIONS*

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1. Introduction

Threonine deaminase (E.C.4.2.1.16) isolated from various bacterial species and yeast behaves as an allosteric protein towards its amino acid ligands, threonine, valine, isoleucine. Positive or negative homotropic and heterotropic effects have been described [1–9].

Some recent observations carried out with different bacterial enzymes tended to restrict the manifestation of cooperativity of threonine molecules to special conditions. Hatfield and Umbarger [10] using cristallized *B. subtilis* threonine deaminase found a sigmoidal response to theonine only when assayed in the presence of negative effector isoleucine. In agreement with Monod, Wyman and Changeux [11] they conluded that the negative effector influences the transition between the two postulated forms of the enzyme. Similar conclusions have been drawn form results on threonine deaminase purified from *B. licheniformis* [9].

Harding [12] using the *E. coli* enzyme in conditions which are quite different from the one defined by Changeux [3] showed that, at pH 8.2, cooperativity of threonine molecules, detectable in the presence of 0.1M phosphate buffer, could be abolished in the presence of 0.5M phosphate buffer. He suggested that the sigmoidal nature of the response in 0.1M phosphate was due to enzyme instability at low substrate concentration and low ionic strength. Quite a similar interpretation was previously presented by Cennamo and Caretti [13] using the enzyme from *Salmonella typhymurium.*

As far as the yeast enzyme is concerned Cennamo et al. [5] noticed that a 150 fold dilution results in an rapid inactivation which can be prevented partly or completely, by addition of saturating amounts of either one, or mixtures, of the following ligands: L-threonine, L-valine, ammonium ions.

Work from this laboratory on yeast threonine deaminase has been carried out with various technics. Extracts were made either in 0.1M or 1M phospate, pH 7.2 or in 1M Tris, pH 8.0 or 8.5. For special purpose 1M KCl was sometimes added to those buffers. Previously, the coupled assay was devised by Holzer et al. [14] was used; more recently we have switched to the colorimetric assay (Friedemann and Haugen [15]). In all these conditions, the allosteric behavior of yeast threonine deaminase was found identical. It has been pointed out however, that yeast threonine deaminase, being not a very stable enzyme, needs special conditions to be kept and becomes very unstable after purification (refs. [7,16] and unpublished results).

It was then conceivable that some enzyme inactivation might have been occurring in all above conditions during incubation of yeast threonine deaminase when subsaturating concentrations of substrate were used and could, partly or completely, account for the positive homotropic effect of threonine already described [7].

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Table 1
Percent of original threonine deaminase activity remaining following preincubation of enzyme with subsaturating concentrations of substrate.

Exp.	L-threonine mM				
	0	1	2	3	4
1	82.4	82.0	82.1	84.0	83.5
2		58.9			61.6
3		76.7			75.0
4		71.1			67.7

Conditions are described under Experimental.

The present report is concerned with a reinvestigation of this problem. The results we have obtained show, that despite partial enzyme inactivation during preincubation with subsaturating substrate concentrations, the yeast enzyme retains its allosteric properties, including the positive homotropic effect of threonine.

2. Experimental

Most of the experiments were carried out with crude extracts of *Saccharomyces cerevisiae* (strain M14R1, previously described[7]). Preparation of cells and extracts was essentially as already described [16].

Preincubation and incubation were performed in Tris buffer 0.2M pH 8.5, containing ammonium sulfate 3×10^{-3}M for optimal activity [16]. Threonine and/or valine were added as described in the legends. The preincubation mixtures were brought to 28°C. Reaction was initiated by addition of cold enzyme preparation. Depending on the extract, 15 to 20 μl (containing 20 to 30 mg protein/ml) were used, the amount of which yielded 50 to 60 μMoles of α-ketobutyrate per minute and per ml final incubation mixture, at substrate saturation. When preincubation and incubation took place the preincubation period of 10 min was terminated in a set of tubes by addition of acid and subsequent determination of the α-ketobutyrate produced. In a second set of tubes additions of either a saturating concentration of L-threonine or L-valine (9mM final concentration) were followed by a 10-min reaction period after which the total α-ketobutyrate produced was estimated. The amount of α-ketobutyrate produced during the preincubation period was subtracted from the total amount formed. All measurements were performed in duplicate. Activities have been expressed as percent of the activity of the enzyme preparation, measured directly at substrate saturation in each experiment.

Some experiments were carried out using a purified threonine deaminase obtained from commercial Baker's yeasts (Springer, France) by a procedure already described [7], modified by the addition of 25mM DL-allothreonine to all buffers and reagents.

3. Results

The first series of experiments were carried out to determine if yeast threonine deaminase is inactivated during incubation in the presence of low concentrations of threonine. The results of four separate experiments are summarized in table 1. In all these experiments an average inactivation of 24%, independent of threonine concentration, was observed. This inactivation is significant but it seems unlikely that the sigmoidal response to threonine can be accounted for by this inactivation. Actually, if this were the case, the inactivation should be threonine dependent.

This is further demonstrated by the following experiments where we reinvestigated the sigmoidal part of the curve in the presence(curve B, fig. 1) and in the absence (curve A, fig. 1) of the modifier, valine. Even if the values of curve A are increased by 24% to compensate for the inactivation, the values of curve B are drastically higher than those expected if valine served only to stabilize enzyme activity. It appears that the effect of valine is heterotropic as described by Changeux [3].

That the valine effect is indeed more than stabilization is shown by the experiments in curves D and E. For curve D, we preincubated the enzyme with 9mM valine for 10 min before the addition of low threonine concentrations. For curve E, the first preincubation was carried out at low substrate concentrations and then 9mM valine was added for an additional 10-min incubation. If we compare curves D and E to curve C which is the theoretical curve deduced from curve B by a 24% inactivation, we can

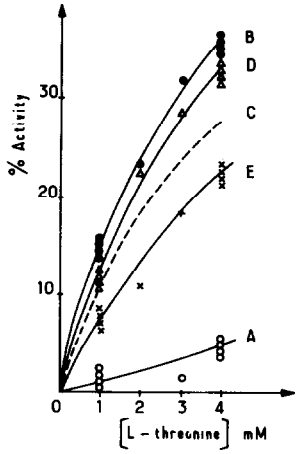

Fig. 1. Differences in stability of threonine and valine fixation sites on yeast threonine deaminase. (A). Direct measurements at low concentrations of substrate. (B). Direct measurements at low concentrations of substrate in the presence of 9mM L-valine. (C). Theoretical curve accounting for 24% inactivation during preincubation. (D). Activity found after 10-min preincubation with 9mM valine followed by addition of low substrate concentration and a second 10-min incubation. (E). Activity found after preincubation with low substrate concentrations, followed by addition of 9mM valine and a second 10-min incubation period.

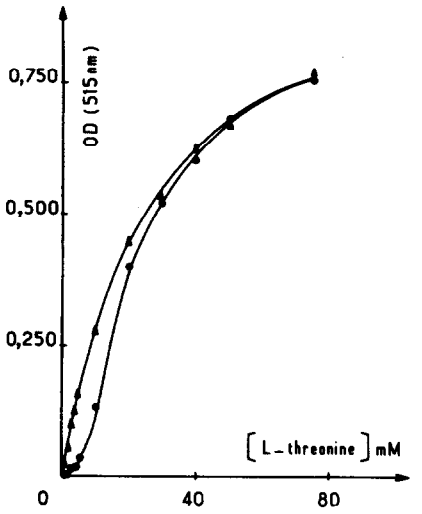

Fig. 2. Allosteric part of the substrate saturation curve for a partially purified threonine deaminase (Baker's yeast). o——o: Threonine alone. Δ——Δ: Threonine + 9mM valine.

conclude: (1) as curve D is higher than curve C, valine has a protective effect and (2) as curve E is lower than curve C, it is an indication that the valine fixation sites are more sensitive to preincubation in low threonine concentrations than the threonine fixation sites. This observation could be explained either by a more rapid destruction of valine sites, or by a loss of affinity of these sites towards valine. The second hypothesis was eliminated after finding that increased valine concentrations (18mM and 36mM instead of 9mM) did not improve the recovery of valine sites. It is then concluded that the number of reactive valine sites decreases more rapidly, in the conditions used, than the number of threonine sites.

The isoleucine attachment sites also appear to behave independently under our preincubation conditions. Thus, under conditions which partially inactivate threonine and valine sites, the activity continues to be 100% inhibited by isoleucine which means these sites remain intact and are more resistant to inactivation than the threonine or valine sites (as stated elsewhere using heat inactivation [7]).

Although, collectively, this data points to the fact that sigmoidal kinetics are the result of cooperative effects of substrate rather than inactivation, Hatfield and Umbarger [10] have shown that the presence of isoleucine was necessary to observe cooperativity with purified threonine deaminase from *B. subtilis*. Since our crude extracts might contain low amounts of isoleucine a partially purified preparation of yeast threonine deaminase was reexamined and, as shown in fig. 2, retained the cooperativity of threonine molecules. Nevertheless, all the purification steps were carried out using a "protective" buffer which contained 25mM DL-allothreonine. Although the dilution of the enzyme in the final assay mixture was enough to exclude inhibition by DL-allothreonine, it remains possible that some of the observed allosteric effects may be due to the presence of this substrate analogue. Another purification carried out in the absence of allothreonine should allow us to examine this possibility.

In summary, all our observations, in all the conditions tried so far, indicate that the sigmoidal response to the substrate, threonine, is the result of an allosteric effect, and thet yeast threonine deaminase exists in a transition equilibrium between two forms (R and T) of the enzyme.

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