DISSOCIATION-PRODUCED LOSS OF REGULATORY CONTROL OF HOMOSERINE DEHYDROGENASE OF RHODOSPIRILLUM RUBRUM *

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1. Introduction

HDH *** of *Rhodospirillum rubrum* is subject to feedback control by several amino acid end products of the pathway in which it functions [1]. Evidence for the reversible aggregation of the enzyme under the influence of ligands has come from density gradient and gel filtration experiments [2]. In this paper we present kinetic data which indicate that the enzyme undergoes a reversible dissociation to a form which is insensitive to feedback inhibition by threonine, as well as to inhibition by the threonine analog β-hydroxynorvaline, and which is also insensitive to activation by methionine and isoleucine. The equilibrium is shifted toward the sensitive form by preincubation with these ligands, as well as certain cations and anions, and toward the modulator-insensitive form by homoserine and by serine, an inhibitory substrate analog.

2. Methods

DL-ASA was prepared from DL-allylglycine [3] with the elimination of contaminating aspartic acid by Dowex AG 50W-X8 (H^+) chromatography.

Buffer A contained 30 mM potassium phosphate, 1 mM K_3EDTA and 0.1 M KCl, pH 7.2, μ = 175 mM.

The standard enzyme assay was in 3.1 ml of buffer A containing 15 μM TPNH and 58 μM L-ASA at 23° in a Cary model 11 spectrophotometer with a 0 to 0.1 absorbancy slide wire and a 1 cm light path. One unit of enzyme is defined as the amount catalyzing the oxidation of 1 μmole of TPNH under these conditions. L-threonine, when added to the assay cuvette, was at a concentration of 0.48 mM. Protein was determined by the method of Lowry et al. [4].

R. rubrum (a gift of Dr. H.Gest) was grown photosynthetically under anaerobic conditions in a synthetic malate medium [1]. The cells were harvested after 48–60 hr of growth. The enzyme was extracted by sonication and purified about 16-fold before use by ammonium sulfate fractionation and was in buffer A.

3. Results

Dilution of the enzyme solution produced a time-dependent decrease in its sensitivity to threonine inhibition (fig. 1). Dilution was with buffer A so that there was no change in salt composition or concentration. It may be noted that the initial relative rate of desensitization was independent of enzyme concentration, indicating a first order reaction.

Resensitization could be achieved by preincubation of the desensitized enzyme with threonine (fig. 2). The resensitization reaction was biphasic and the

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*** Abbreviations: HDH, homoserine dehydrogenase; ASA, aspartic-β-semialdehyde.

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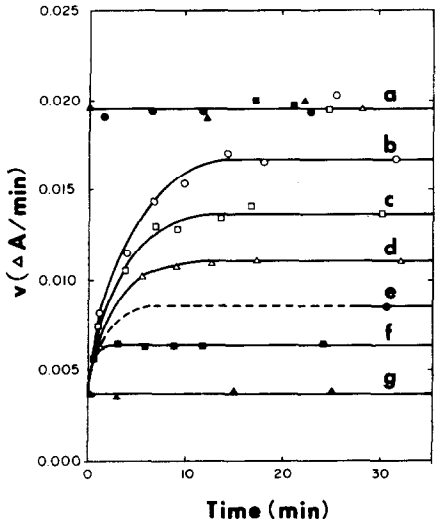

Fig. 1. Desensitization of HDH to threonine inhibition by enzyme dilution. Enzyme solution (9.5 units/ml) was diluted with buffer A as indicated below and incubated at 23°. Curve a is the activity in the absence of threonine, and curves b through g are activities in the presence of threonine of enzyme diluted 1000-fold, 300-fold, 100-fold, 30-fold, 10-fold and undiluted, respectively. One μl of the undiluted enzyme was used for assay and correspondingly larger aliquots of diluted enzyme so that the total enzyme in the assay cuvette was constant. The symbols in curve a refer to the dilutions in curves b through g with corresponding symbols.

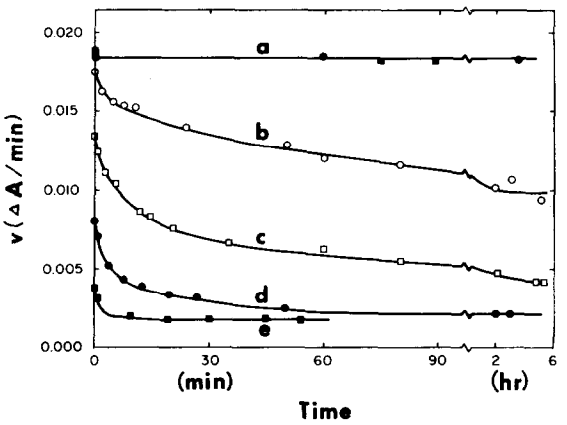

Fig. 2. Resensitization by threonine of HDH previously desensitized by enzyme dilution. Enzyme (9.5 units/ml) was desensitized by dilution as in fig. 1. L-threonine solution was then added to produce a final concentration of 0.5 mM and the solution further incubated at 23°. Quantities of enzyme taken for assay were as in fig. 1. Curve a is the activity in the absence of added threonine. Curves b through e are the activities in the presence of threonine of enzyme diluted 3000-fold, 300-fold, 30-fold and undiluted, respectively. Zero time points were assays after desensitization but before addition of threonine. The symbols in curve a refer to the dilutions in curves b through e with corresponding symbols.

initial rate increased with increasing enzyme concentration. It may be noted by comparison with curve g, fig. 1, that in curves d and e of fig. 2 the enzyme was sensitized beyond the original level.

Sensitivity to threonine, with and without preincubation with threonine, is plotted as a function of enzyme concentration in fig. 3. The plateau value in the preincubated curve is the maximum sensitivty under the test conditions when the enzyme is fully in the sensitive form. It is apparent that the curve for unpreincubated enzyme is approaching the fully sensitized and desensitized states with increasing and decreasing enzyme concentrations, respectively.

The curve of degree of resensitization as a function of the concentration of L-threonine in the preincubation mixture showed a distinct sigmoidicity with a Hill coefficient of 2.2.

Reduction of the salt concentration of the enzyme solution also produced a time-dependent decrease in the sensitivity of the enzyme to threonine inhibition, which could be reversed by readdition of salt. The initial rate of resensitization again increased with increasing enzyme concentration.

The activators of the enzyme, isoleucine, methionine and norleucine [1], and the inhibitory threonine

Table 1
Activation by isoleucine of homoserine oxidation

Enzyme form	Activity (ΔA/min)		% activation
	− isoleucine	+ isoleucine	
associated	0.0043	0.0098	128
dissociated	0.0018	0.0020	10

Assay mixture contained 30 mM Tris, pH 8.3, 0.1 KCl, 1 mM EDTA, 4.8 mM L-homoserine, 125 μM TPN, 2.1 mM L-isoleucine (when added) and 5 μl of associated enzyme (11 units/ml) or 75 μl of dissociated enzyme (0.32 units/ml).

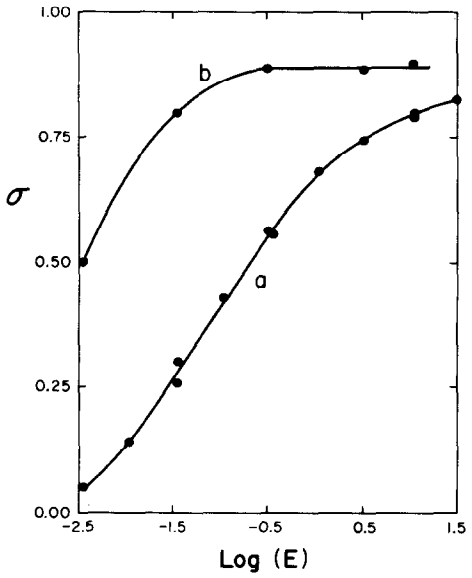

Fig. 3. Sensitivity of HDH to threonine inhibition as a function of enzyme concentration before and after preincubation with threonine. Sensitivity (σ) is defined as $[1 - v_i/v_0]$, where v_0 and v_i are the velocities in the absence and presence of threonine, respectively, measured after equilibration of the preincubation solution. Total concentration of enzyme (E_t) is in units/ml. Curves a and b represent enzyme before and after preincubation with 0.5 mM L-threonine (cf. fig. 2).

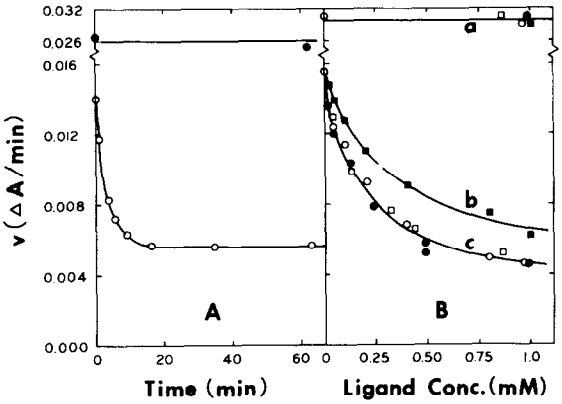

Fig. 4. Resensitization by isoleucine, methionine, β-hydroxynorvaline and norleucine of enzyme previously desensitized by enzyme dilution. Enzyme (14 units/ml) was diluted 100-fold with buffer A for desensitization. (A) L-isoleucine was added to give a final concentration of 0.42 mM and the solution further incubated at 23°. Aliquots (0.1 ml) were removed periodically for assay in the absence (filled circles) and presence (open circles) of threonine. Zero time points were assays after desensitization but before addition of isoleucine. (B) Either L-isoleucine (open squares), L-methionine (filled squares), DL-β-hydroxynorvaline (open circles) or DL-norleucine (filled circles) was added to the final concentration shown (of the L-form). After a time sufficient for equilibration at 23°, aliquots (normalized to 0.1 ml) were removed for assay in the absence (curve a) and presence (curves b and c) of threonine.

analog, β-hydroxynorvaline, produced a resensitization of the enzyme analogous to the effect of threonine (fig. 4). The dissociation-produced loss of activatability of the reverse reaction [1] is shown in table 1 for isoleucine.

Homoserine and serine produced a partial desensitization of the enzyme, while ASA, TPN and TPNH had no effect. Inhibition by serine, in contrast to that with threonine and β-hydroxynorvaline, was virtually independent of the state of the enzyme. An additional distinction was that serine inhibition was not reversed by isoleucine or methionine.

4. Discussion

The conclusion seems inescapable from these results that the modulator-sensitive and insensitive forms of homoserine dehydrogenase arise from an association-dissociation reaction. The ability of modulators to shift the equilibrium to the aggregated state indicates that they do not bind to dissociated species, thus apparently ruling out a dissociation into distinct active and regulatory subunits, as in the case of *p*-mercuribenzoate-treated aspartic transcarbamylase [5].

The loss of sensitivity to the threonine analog β-hydroxynorvaline, in parallel to the loss of sensitivity to threonine itself, identifies it as an allosteric ligand, presumably sharing binding sites with threonine. On the other hand, inhibition by the substrate analog, serine, is conserved with dissociation, as is activity, identifying serine as an isosteric ligand.

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