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ENHANCED ALLOSTERIC REGULATION OF THREONINE DEAMINASE AND ACETOHYDROXY ACID SYNTHASE FROM *ESCHERICHIA COLI* IN A PERMEABILIZED-CELL ASSAY SYSTEM

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Summary

A permeabilized-cell technique for rapid assay of enzyme activity has revealed enhanced allosteric regulation of both threonine deaminase (L-threonine hydrolyase (deaminating), EC 4.2.1.16) and acetohydroxy acid synthase (acetolactate pyruvate-lyase (carboxylating), EC 4.1.3.18) in *Escherichia coli* K-12. In the permeabilized cell assay threonine deaminase exhibited a higher Hill coefficient for inhibition by L-isoleucine, and acetohydroxy acid synthase exhibited a hypersensensitivity to allosteric inhibition by L-valine when compared to studies on crude extracts. We propose that these effects reflect the in situ microenvironments of both enzymes. Preliminary evidence further indicates that acetohydroxy acid synthase may loosely associate with the cell membrane.

Introduction

Assay systems for both threonine deaminase (L-threonine hydro-lyase (deaminating), EC 4.2.1.16) and acetohydroxy acid synthase (acetolactate pyruvate-lyase (carboxylating), EC 4.1.3.18), employing cetyltrimethylammonium bromide as a cell-permeabilizing agent, have been developed and utilized for mass screening of valine-resistant mutants of *Escherichia coli* K-12 [1]. Control experiments to establish the validity of threonine deaminase- and acetohydroxy acid synthase-permeabilized cell assays, without pre-treatment of cells with cetyltrimethylammonium bromide, revealed that acetohydroxy acid synthase was markedly more sensitive to inhibition by low concentrations of valine than in clarified crude extracts. Subsequent experiments suggest that the altered

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inhibitability of acetohydroxy acid synthase is probably not an artifact of the assay system, but rather reflects the microenvironment of that enzyme in situ. We postulate that an interaction with the cell membrane may be part of this in situ microenvironment. The sigmoidicity of the isoleucine inhibition curve for threonine deaminase was enhanced in the permeabilized-cell assay system. This supports our interpretation that the permeabilized-cell assay system may constitute a more physiological state for the enzyme. A portion of this work has been described previously [2].

Methods

All experiments were performed using *E. coli* strain MJ6 (K-12, *rbs-215*, also known as CU2001), obtained from H.E. Umbarger (Department of Biological Sciences, Purdue University). Culture conditions, preparation of extracts by sonic oscillation, and enzyme assay methods for acetohydroxy acid synthase were as described previously [1]. Threonine deaminase was assayed similarly with the following modifications: 0.15 mM L-isoleucine in 0.1 M potassium phosphate (pH 8.0) was used for cell washing; the assay reaction mixture was appropriate for threonine deaminase [3]; 0.4 ml 0.1 M Tris · HCl (pH 8.0)/0.15 mM L-isoleucine was used for the final resuspension of cells from each 10 ml volume of culture grown to a Klett reading of 150 (number 42 filter) to be used for assay or extract preparation. Protein concentration was measured by the Biuret method [4]. All manipulations except for growth and turbidity measurements, were made at 0—4°C. Assay results were normalized to reaction periods of 20 min for threonine deaminase and 10 min for acetohydroxy acid synthase. All chemicals were of reagent grade or were the highest purity commercially available. Cetyltrimethylammonium bromide was obtained from Sigma Chemical Co.

Kinetic parameters of inhibition and reactivation experiments were estimated by FORTRAN computer programs adapted from those of Cleland [5,6]. These performed least squares fits of percent inhibition (or reactivation) versus effector concentration data, either to a rectangular hyberbola (Michaelis-Menten equation) or to the Hill equation. Programs HYPER and HILL were obtained from W.W. Cleland, University of Wisconsin, through H.E. Umbarger, Purdue University. We have defined, for comparative use, the effector concentration giving half-maximal inhibition (or reactivation) as the $I_{0.5}$ (or $R_{0.5}$), and the maximum percent inhibitability (an asymptotic value) as the I_{max}. In some cases, the $I_{0.5}$ approaches the true value of the inhibition constant, K_i, since all inhibition experiments were performed with a substrate concentration exceeding the K_m by at least 10-fold.

Optimum yield of threonine deaminase activity in the permeabilized-cell system was obtained at a concentration of 0.0071% cetyltrimethylammonium bromide employing the standard cell concentration defined above. This surfactant was combined with assay components prior to addition of cells. The cell suspension was not treated with surfactant prior to assay. Under these conditions, threonine deaminase activity was proportional both to cell density and time of assay, up to 30 min (unpublished data). At lower or higher cell densities, correspondingly lower or higher surfactant concentrations were optimally

effective, and gave results consistent with the expected level of enzyme activity. Similar results were obtained with acetohydroxy acid synthase, except that the assay was linear with respect to time for 20 min at 37°C. Maximum effector sensitivity for both enzymes appeared comparable to that seen in crude extracts.

Cetyltrimethylammonium bromide only slightly decreased threonine deaminase activity in crude extracts during the course of assay, while it often caused a partial loss of acetohydroxy acid synthase activity in crude extracts, especially in the absence of membrane and cell wall material (Table I). This is not unusual behavior for an ionic surfactant [7]. However, when added in an optimal ratio to cell density, cetyltrimethylammonium bromide may have bound primarily to the cell membrane and wall, leaving a relatively low concentration to bind with deleterious effects to the enzyme being studied. Additionally, the enzyme may have been stabilized by the cell membrane/wall fraction.

Relative enzyme activity in the permeabilized-cell assay system, as compared to that released by sonic disruption, was about 107% for threonine deaminase and 89% for acetohydroxy acid synthase, on an equivalent cell density basis. Thus, detection of enzyme activity by either method was comparably efficient. Destabilization of acetohydroxy acid synthase by the surfactant in the absence of a complete assay mixture precluded our obtaining a good estimate of the fraction of enzyme retained inside surfactant-treated cells.

Results

Having observed unexpected hypersensitivity of acetohydroxy acid synthase to inhibition by valine in preliminary experiments, we sought confirmation in a more detailed study on crude extracts (Table I). The valine concentration giving half-maximal inhibition (the $I_{0.5}$ for valine) in the permeabilized cell assay was in the range 15—30 μM, about one-sixth of the value normally obtained in clarified crude extracts (Table I this paper and Fig. 3 in ref. 8). The hypersensitivity to valine was not due to a direct effect of surfactant on the enzyme, nor to an enhancement of inactivation of the enzyme, since the $I_{0.5}$ of soluble acetohydroxy acid synthase remained unaltered (Table I) and the assay remained linear with respect to time in the presence of both surfactant and valine.

The hypersensitivity of acetohydroxy acid synthase to inhibition by valine is not likely to have been caused by anomalously high valine concentrations inside the surfactant-treated cells. Direct measurement of valine concentration was not feasible since the mixture of cell suspension and surfactant did not filter well through Millipore filters. However, if active transport mechanisms for the branched-chain amino acids [9,10] were still functional to concentrate valine, then it is likely that the isoleucine concentration within the permeabilized cells would also be high. Fig. 1 shows inhibition curves for threonine deaminase in a clarified crude extract compared with the permeabilized-cell system. The $I_{0.5}$ for isoleucine was higher in the permeabilized-cell system and the value of the Hill coefficient, n (Fig. 1), was increased. The higher $I_{0.5}$ observed in the permeabilized cells would not occur as a consequence of an

TABLE I

EFFECT OF CETYLTRIMETHYLAMMONIUM BROMIDE ON ACETOHYDROXY ACID SYNTHASE IN A CRUDE SONIC EXTRACT

Fraction	Additions to assay	Relative enzyme activity [a]	$I_{0.5}$ for valine [b] ±S.E. (μM)	I_{max} [b] ±S.E. (%)
Whole sonic extract	None	1.24	92.1 ± 5.8	89.1 ± 1.8
Whole sonic extract	Surfactant [d]	1.85	38.0 ± 2.9	87.7 ± 1.7
Clarified extract	None	1.00	97.4 ± 9.4	89.1 ± 2.8
Clarified extract	Surfactant	0.71	85.8 ± 8.7	75.6 ± 2.4
Washed pellet [c]	None	0.10	n.d.	n.d.
Washed pellet	Surfactant	0.70	15.5 ± 1.5	79.9 ± 1.4

[a] Data shown represent typical distribution of acetohydroxy acid synthase activity. Replicate experiments differed from each other within a maximum ±12% range. The normalized value, 1.00, corresponds to a specific activity of 25.1 nmol of acetolactate formed/min per mg protein.

[b] Calculated by assuming a hyperbolic saturation curve for percent inhibition as a function of valine concentration [1].

[c] Includes whole cells and cell surface fragments.

[d] Cetyltrimethylammonium bromide.

n.d., not determined.

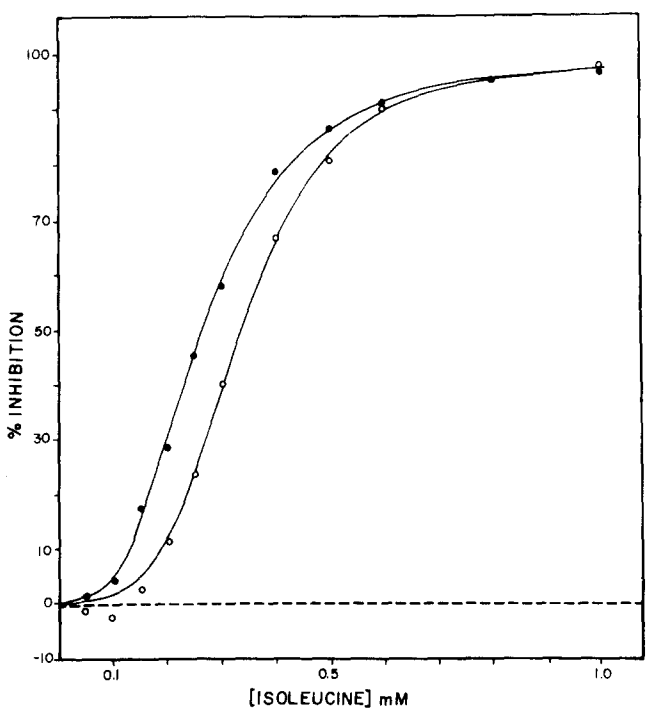

Fig. 1. Effect of L-isoleucine on threonine deaminase, comparing a crude extract with permeabilized cells. ○, permeabilized-cell assay; ●, crude extract assay. The solid lines represent theoretical curves derived from computer-fitted values for the inhibition parameters (excluding the points showing activation). These values are $I_{0.5}$ = 0.333 ± 0.006 mM, K_i = 0.121 ± 0.0017 mM, and n = 4.01 ± 0.11 for permeabilized cells; $I_{0.5}$ = 0.264 ± 0.007 mM, K_i = 0.0169 ± 0.0028 mM, and n = 3.06 ± 0.11 for the crude extract.

elevation of the intracellular isoleucine concentration, but rather a lower $I_{0.5}$ would be expected. The increased value of n may reflect a greater degree of subunit interaction in the permeabilized-cell system than in crude extracts. Further, the more fundamental binding parameter, K_i (calculated as $I_{0.5}{}^{1/n}$), is the same for both systems. In contrast, the relatively weak inhibition of acetohydroxy acid synthase in crude extracts by high concentrations of isoleucine (2—10 mM) was greater in the permeabilized-cell system by as much as 50% (e.g., 5 mM isoleucine inhibited enzyme activity 50% in a crude extract, while it inhibited enzyme activity 81% in the permeabilized cell system).

Concentrations of isoleucine in the range 0.05—0.15 mM resulted in a slight activation (1—3%) of threonine deaminase in contrast to the inhibition seen at higher concentrations. This was not observed in crude extracts. Such behavior has been seen with threonine deaminase from other organisms [11—15] in crude extracts as well as with purified enzyme preparations, though only variably in *E. coli* [16—18].

A more direct test of the possibility of intracellular valine accumulation employed isoleucine-inhibited threonine deaminase. Isoleucine-inhibited threonine deaminase can be reactivated by valine in a concentration-dependent fashion. A comparison of this valine antagonism of isoleucine inhibition revealed no significant difference between the two assay systems (Fig. 2). We conclude that valine does not appreciably accumulate in surfactant-treated cells. The hypersensitivity of acetohydroxy acid synthase to inhibition by valine was retained in the presence of isoleucine (Fig. 3), which indicated that the results obtained from reactivation of threonine deaminase by valine were

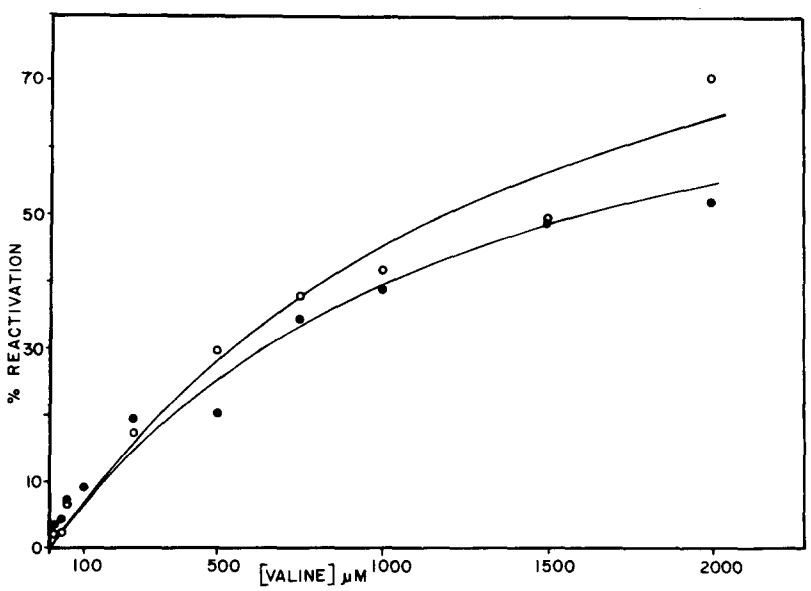

Fig. 2. Reactivation by L-valine of threonine deaminase inhibited by 0.3 mM L-isoleucine. Notation as in Fig. 1. The fitted values for $R_{0.5}$ are 1.24 ± 0.20 mM for permeabilized cells and 1.53 ± 0.25 mM for the crude extract. Percent reactivation (R) was calculated according to the formula, $R = [100(I_o - I)]/I_o$ where I_o is the initial percent inhibition in the presence of 0.3 mM L-isoleucine, and I is the observed percent inhibition by 0.3 mM L-isoleucine in the presence of a given concentration of L-valine.

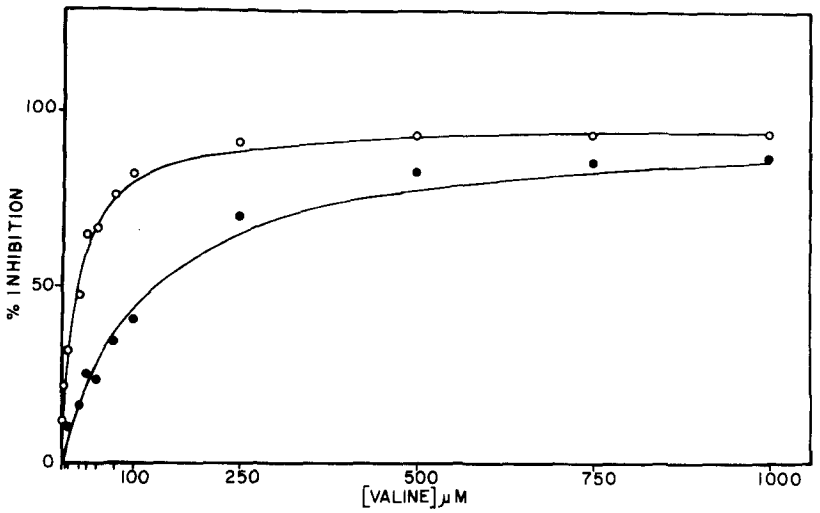

Fig. 3. Inhibition of acetohydroxy acid synthase by L-valine in the presence of 0.3 mM L-isoleucine. Notation as in Fig. 1. The computer-fitted values for $I_{0.5}$ are 19.7 ± 1.6 μM for permeabilized cells and 115.9 ± 12.2 μM for the crude extract.

not influenced by isoleucine inhibition of valine accumulation.

The possibility remained that substrate exclusion in the permeabilized cell system might account for an apparent hypersensitivity of acetohydroxy acid synthase to inhibition by valine. However, the K_m of the enzyme for pyruvate in the permeabilized cells was 1.81 ± 0.31 mM and in crude extracts was 1.56 ± 0.18 mM. Both values are the average of two separate experiments ± standard deviations and are not significantly different from each other. We therefore conclude that no significant exclusion of pyruvate occurred. The numerical results compare favorably with the K_m (3.3 mM) calculated from Umbarger and Brown (Fig. 3 in ref. 8).

TABLE II

INHIBITION OF ACETOHYDROXY ACID SYNTHASE BY VALINE IN FRACTIONS FROM A SONIC EXTRACT

The fractions below were prepared according to an adaptation of the method of Futai [19]. A washed suspension of cells in stabilizing buffer [1] (5 ml per g wet wt. of cells from a 4 l culture harvested at a Klett reading of 150 using a number 42 filter) was sonified for 8 min (sixteen 30-s bursts with intervening 1-min rest periods) at a power level of 71 W, until the turbidity of the cell suspension was 5% of its initial value. After centrifugation at 8000 × *g* for 10 min to sediment unbroken cells, the supernatant extract was centrifuged at 100 000 × *g* for 90 min. The pellet of membrane material was resuspended in 1/27 volume of stabilizing buffer. Both supernatant and membrane suspension were then assayed for enzyme activity. Initial experiments employing a French pressure cell (Aminco) to disrupt the bacterial cells yielded comparable results.

	No surfactant		+ Cetyltrimethylammonium bromide	
	Membrane pellet resuspension	High-speed supernatant	Membrane pellet resuspension	High-speed supernatant
$I_{0.5}$ (μM)	71.0 ± 6.2	174.7 ± 18.7	54.4 ± 7.9	125.7 ± 24.7
I_{max} (%)	84.4 ± 2.3	99.7 ± 4.2	77.4 ± 3.2	91.5 ± 6.6

Since tests for altered ligand and substrate binding revealed little basis for the hypersensitivity of acetohydroxy acid synthase to valine in the permeabilized cell assay, we examined the possibility of interaction with the cell membrane. Although very little acetohydroxy acid synthase activity (approx. 1%) sedimented with cell-free membranes as a result of high-speed centrifugation, the enzyme activity in a suspension of an unwashed membrane pellet (Table II) had an enhanced sensitivity to inhibition by valine that was independent of cetyltrimethylammonium bromide. This is similar to the enhanced sensitivity seen in the permeabilized cell assay system. Note that the behavior of the cell-free membrane suspension (Table II) was quite different from that of the crude particulate fraction (Table I) which was composed primarily of unbroken cells. Cetyltrimethylammonium bromide did not significantly change the kinetic parameters for the inhibition observed (Table II).

Discussion

It appears that unique macromolecular interactions, disrupted upon preparation of a crude extract, may be responsible for the observed hypersensitivity of acetohydroxy acid synthase to inhibition by valine. Since we have obtained enhancement of inhibitability of acetohydroxy acid synthase retained in a cell membrane fraction, we postulate that these macromolecular interactions are with the cell membrane, perhaps with a transport system [9,10]. Enzymes normally associated with membranes have been observed to undergo changes in properties when in the soluble state [20,21]. It should be noted that de Felice et al. [22] may have observed a link between acetohydroxy acid synthase and the cell membrane in the case of *ilvH* (*brnP*) mutants. These mutants exhibited both a decrease in sensitivity of acetohydroxy acid synthase to inhibition by valine as well as inhibition of branched-chain amino acid transport. However, the authors interpreted the inhibition of transport to result from an increased intracellular pool of valine. It is nonetheless conceivable that transport properties were altered as a direct consequence of the change in acetohydroxy acid synthase structure. The relationship of the membrane-association hypothesis to the known multiplicity of acetohydroxy acid synthase activities in *E. coli* [1,22—26] is not certain. Experiments to establish the nature of the macromolecular interactions we propose must await purification of the enzyme.

We have considered several alternate possibilities to our explanation to account for the behavior of acetohydroxy acid synthase under conditions of this permeabilized cell system: (1) a high local enzyme concentration within "leaky bags" (surfactant-permeabilized cells) might account for an apparently higher valine sensitivity of the enzyme; (2) a high local inhibitor concentration would make the measurable enzyme activity lower and would cause an apparently increased sensitivity to small increases in effector concentration; and (3) cetyltrimethylammonium bromide may be entirely responsible for apparent enzyme changes.

Changes in enzyme concentration alone cannot account for the different effector sensitivities of acetohydroxy acid synthase since no enzyme concentration-dependent increases in sensitivity to inhibition by valine occur over the 10-fold range normally employed in assay of the enzyme. Moreover, the aceto-

hydroxy acid synthase activity in membrane preparations (Table II) was low, yet was still hypersensitive to inhibition by valine. Since recent experiments on highly dilute crude extracts (1/100 of the normal concentration range 4—8 mg protein per ml) have revealed a reversible desensitization of acetohydroxy acid synthase (Patterson, E.B. and Blatt, J.M., unpublished data), the tendency to hypersensitivity in membrane preparations assumes greater significance than the numerical results would indicate. The low concentration of acetohydroxy acid synthase activity in membrane preparations would be expected to be less, rather than more, sensitive to valine.

Accumulation of valine has been ruled out since threonine deaminase responds to valine identically in both the permeabilized-cell system and in crude extracts. Substrate exclusion has been ruled out by the near identity of K_m for pyruvate in both assay systems. A direct effect of cetyltrimethylammonium bromide on the enzyme is similarly unlikely. The results in Table I show no effect of that surfactant on the kinetic parameters for inhibition of aceto-hydroxy acid synthase in the clarified supernatant fraction of a crude sonic extract. The surfactant served only to reveal activity which was sequestered in unbroken cells, and the hypersensitivity of that activity is consonant with the results obtained from surfactant-aided assay of activity in otherwise untreated cell suspensions. Similarly, cetyltrimethylammonium bromide did not signifi-cantly alter the kinetic parameters shown in Table II. Supernatant activity, as well as activity resident in the partially purified membrane fraction, was unaffected by the presence or absence of the surfactant. Therefore, neither the surfactant nor a surfactant · membrane complex is likely to have caused the observed enzyme changes.

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