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CONFORMATIONS OF LYSINE-SENSITIVE ASPARTOKINASE

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Summary

1 The technique of differential thermal and proteolytic inactivation has been employed as a conformational probe for the lysine-sensitive aspartokinase (EC 2 7 2 4) of *Escherichia coli* B

2 L-Amino acid inhibitors of this enzyme each induce a characteristic enzyme conformation This is evidenced by rates of thermal and proteolytic inactivation and Arrhenius activation energies for thermal inactivation which are characteristic of the amino acid present

3 Phenylalanine and leucine binding are mutually exclusive as evidenced by competitive behavior in thermal inactivation experiments, suggesting a hydrophobic amino acid binding site with broad specificity

4 The phenylalanine-dependent conformation and the leucine-dependent conformation differ considerably In comparison with the native enzyme, the former is more labile to proteolysis by trypsin whereas the latter is more stable First-order rate constants for thermal inactivation of the phenylalanine- and leucine-dependent conformations are, respectively, about one-half and one-tenth that of the native enzyme

5 Items 3 and 4 taken together suggest that the conformations are ligand induced and do not arise via ligand stabilization of spontaneously arising conformers

Introduction

The activity of several bacterial aspartokinases (EC 2 7 2 4) is altered by hydrophobic amino acids [1—5] including one activated by leucine in *Bacillus subtilis* [1] With the lysine-sensitive kinase of *Escherichia coli*, several hydrophobic amino acids display cooperative heterotropic interactions with lysine [2] On the other hand, with aspartokinases subject to concerted feed back inhibition in a thermophilic bacterium, in *Rhodospirillum tenue*, and in

Bacillus polymyxa, hydrophobic amino acids reverse inhibition by lysine and/or threonine [3—5] The metabolic significance of these effects is not understood but it clearly involves modulation of the enzymes response to primary end-product control

The inhibition of the lysine-sensitive kinase of *E coli* B by hydrophobic amino acids is incomplete, ranging from about 30% at saturating valine concentrations to around 75% at saturating concentrations of phenylalanine (unpublished) These facts, together with the non-competitive nature of lysine inhibition [6,7], suggest that the various amino acids induce or stabilize conformations of the enzyme which have different catalytic capacities

This idea is consistent with the concept that this aspartokinase is a "V" system [8] allosteric enzyme in which V is altered by effectors to a much greater extent than are K_m values Furthermore, the synergy that exists between the hydrophobic amino acids and lysine suggests that the forms of the protein with diminished catalytic capacity have increased affinity for lysine Again, this is consistent with the original hypothesis and with recent binding studies [9]. These considerations have led us to ask the following questions (1) Are there different protein conformations resulting from binding of the various amino acid inhibitors? (2) Do the various hydrophobic amino acids bind at a relatively non-specific site(s) or do separate binding sites exist for each amino acid? We have adopted the method of differential inactivation to gain answers to these questions and present the results in this communication

Methods

Growth of E coli, purification and assay of aspartokinase E coli B cells were grown in continuous culture in a glucose-salts medium at 39°C as previously described [10]. Lysine-sensitive aspartokinase was prepared by previously published procedures [11—13]. Aspartokinase activity was assayed by the hydroxamate procedure as previously described [11] A unit is 1 μmol of aspartylhydroxamate produced per min Both pure and partially purified preparations were used in this study

Thermal inactivation Prior to use in inactivation studies, aspartokinase was chromatographed over a 1 × 15 cm column of Sephadex G-25 equilibrated with 20 mM potassium phosphate, pH 6 75, and 0 1 mM $MgNa_2$ EDTA to remove residual salts and lysine present in the purification buffer After assaying the protein content by absorbance at 225 and 215 nm [14] β-mercaptoethanol was added to a final concentration of 30 mM Aspartokinase (0 2 mg/ml) in 0 05 ml of the above buffer was mixed with 0.05 ml of water or amino acid solution, immersed in a thermostated water bath and after a time, removed, cooled by immersion in an ice bath and the addition of 0 4 ml of the above phosphate buffer Residual enzyme activity remaining in this solution was assayed in triplicate and compared to an identically prepared but unheated control sample Various amino acids and KCl were added to the solution to be heated (as well as its corresponding unheated control) Concentrations, time, and temperatures are given in the figure legends First-order rate constants for inactivation were obtained from the slopes of least square fits of log fractional activity remaining versus time

Proteolytic inactivation Aspartokinase (50 μg/ml) was incubated with trypsin (CalBiochem , 0 33 mg/ml) in phosphate buffer (20 mM potassium phosphate, pH 7 0, 30 mM β-mercaptoethanol, 0 1 mM $MgNa_2$ EDTA) at 25°C At various time intervals, 0 3-ml aliquots were withdrawn from this reaction mixture and mixed with 0 3 ml of a 2 mg/ml solution of soybean trypsin inhibitor (Sigma) in the same buffer to terminate the proteolytic reaction The residual enzyme activity was assayed and compared to a control as previously described for thermal inactivation experiments Trypsin solutions were prepared just prior to use Trypsin was assayed using α-*N*-benzoyl-DL-arginine-*p*-nitroaniline [15] and by this assay, lysine did not inhibit trypsin activity

Results

Thermal inactivation as a function of time is presented in Fig 1 and proteolytic inactivation is similarly presented in Fig 2 Under both conditions, inactivation follows first-order kinetics, irrespective of the presence of saturating concentrations of various amino acids In Fig 1, all L-amino acids tested provided significant protection from thermal inactivation but each produced a characteristic rate at saturation Halving the amino acid concentrations used in Fig 1 did not alter the inactivation rate For example, the rate constants with 40 and 80 mM phenylalanine were 0.73 and 0 71 min^{-1}, respectively Protection from thermal inactivation is also stereospecific Inactivation in the presence of 40 mM D-leucine, 40 mM D-phenylalanine, or 5 mM D-lysine was not significantly different from that observed in the absence of amino acids The rate constants obtained were 1 29, 1 21 and 1 26 min^{-1}, respectively, compared to 1 36 min^{-1} obtained in the absence of any amino acid Corresponding L-

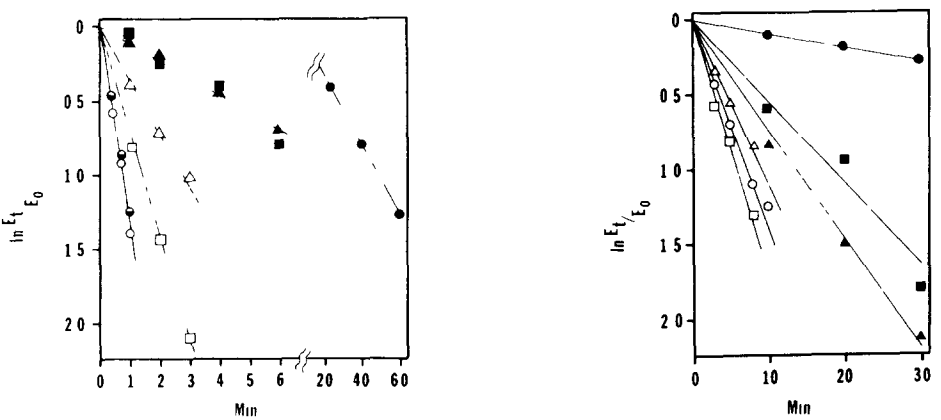

Fig 1 Thermal inactivation of aspartokinase in the presence and absence of saturating concentrations of amino acids ○, no ligands, ■, 40 mM L-leucine, ●, 5 mM L-lysine, ▲, 100 mM L-isoleucine, , 100 mM L-methionine, □, 80 mM L-phenylalanine, ◒, 5 mM D-lysine The temperature was 56°C The aspartokinase specific activity was 13 units/mg

Fig 2 Proteolytic inactivation of aspartokinase in the presence and absence of saturating concentrations of amino acids □, 40 mM L-phenylalanine, ■, 40 mM L-leucine, ●, 5 mM L-lysine, , 50 mM L-methionine, ▲, 50 mM L-isoleucine, ○, no ligand The temperature was 25°C The aspartokinase specific activity was 13 units/mg

amino acids gave values of 0 13, 0 71 and 0 02 min^{-1}, respectively

The rate constants obtained with saturating concentrations of phenylalanine, leucine and lysine are not significantly affected by the purity of the enzyme preparation. In the absence of protective ligands, there is a slight tendency for aspartokinase to become more stable with increasing purity up to a specific activity of about 10 units/mg. Above this, purity does not affect the inactivation rate.

Arrhenius plots for thermal inactivation with saturating ligand concentrations are shown in Fig. 3 Activation energies obtained from these data are E_a, (L-lysine) 164 ± 12 kcal/mol, E_a, (no ligand) 125 ± 4 kcal/mol, E_a, (L-leucine) 206 ± 25 kcal/mol and E_a, (L-phenylalanine) 175 ± 11 kcal/mol Activation energies obtained are characteristic of the saturating ligand and are greater than when no ligands are present However, unless one excludes lysine, there is no parallel relationship between the inactivation rate constant and the activation energy suggesting that the pre-exponential term of the Arrhenius equation contributes significantly to the differences observed in inactivation rates with the various ligands

As shown in Fig 4, the pH optimum for thermal inactivation is not altered by the ligands tested. This rules out the possibility that the difference between ligands is due to their affecting the pH optimum for the denaturation reaction

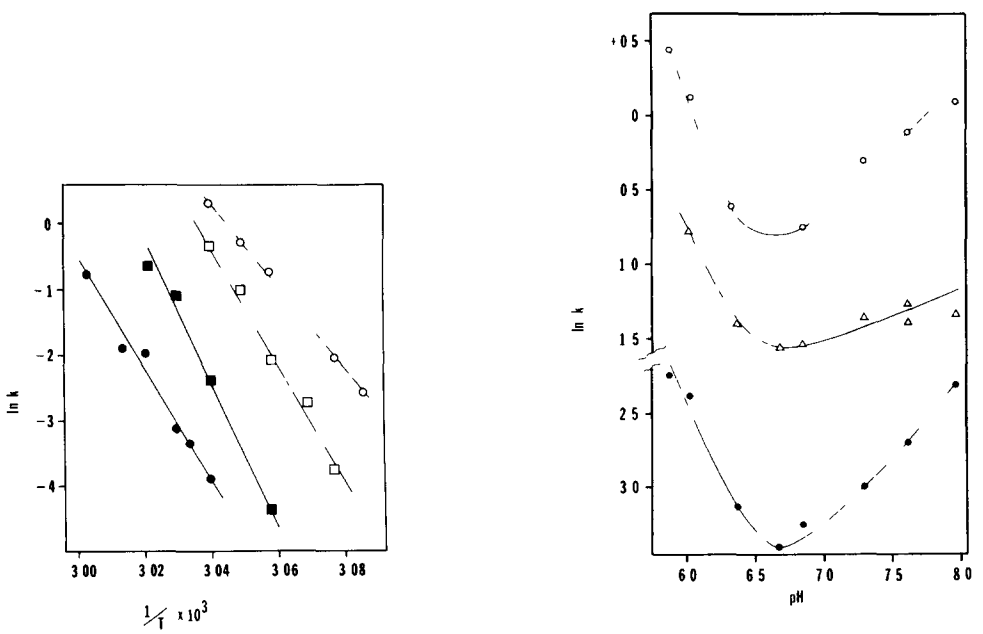

Fig 3 Arrhenius plots for thermal inactivation of aspartokinase in the presence and absence of saturating concentration of amino acids ○, no ligand, ●, 5 mM L-lysine, ■, 40 mM L-leucine, □, 80 mM L-phenylalanine The aspartokinase specific activity was 13 units/mg

Fig 4 pH dependence of thermal inactivation of aspartokinase First-order rate constants for inactivation were determined at 56° C as described in the text except that the buffer pH was varied and 0 24 M KCl was present ○, 0 24 M KCl, △, 80 mM L-phenylalanine and 0 24 M KCl, ●, 40 mM L-leucine and 0 24 M KCl Aspartokinase specific activity was 13 units/mg

The contrast to thermal inactivation, phenylalanine labilized aspartokinase toward proteolytic inactivation Further comparison of Figs 1 and 2 shows that while isoleucine and leucine produce almost identical results in thermal denaturation, they appear different in proteolytic inactivation

When differential inactivation data obtained at sub-saturating lysine concentrations are treated quantitatively according to Citri [16], the "Hill coefficients" obtained are 2 2 and 1 9, respectively, for thermal inactivation and proteolytic inactivation These values agree reasonably well with the generally accepted value of 2 0 for the Hill coefficient for lysine inhibition [2,12,17] and lysine binding to aspartokinase [12] (see ref 9 for a somewhat lower value for the *E coli* $K_{1\,2}$ enzyme)

The experiment in Fig 5 was conducted to gain some information on the question of whether separate sites exist for each of the hydrophobic amino acids The plot shown in Fig 5 is similar to a Dixon plot and it can be shown that for a competitive case (or mutually exclusive binding of the two ligands) the plot of $(k_L - k_P)/(k - k_P)$ versus phenylalanine concentration at different fixed concentrations of leucine intersect at a value of unity on the ordinate and a value numerically equivalent to $-K_p(k_F - k_L)/(k_L - k_P)$ on the abscissa, k_F, k_L, and k_P are thermal denaturation rate constants for free enzyme, leucine-saturated, and phenylalanine-saturated enzyme, respectively k is the observed rate constant for inactivation at various combinations of leucine and phenylalanine K_p is the dissociation constant for the enzyme-phenylalanine complex * This experiment provides support for the idea that the hydrophobic amino acids bind to the same site

Discussion

The conformational mobility of a protein may be explored by a variety of techniques Among these is differential inactivation which has been recently reviewed by Citri [16] While this method is indirect, it is sensitive, and since it relies on measurements of enzyme activity, is a "high information" method compared to more direct physical methods [18] The necessity of correlating physical measurements with enzyme activity is illustrated by the observation that both Li^+ and K^+ induce the same difference spectrum in pyruvate kinase but only K^+ supports catalytic activity [19]

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* The equation derived for Fig 5 is

$$\frac{k_L - k_P}{k - k_P} = \frac{K_1 + L}{K_1 k' + L} + \frac{K_1}{K_1 K_p k' + K_p L} P$$

Where $k' = (k_F - k_P)/(k_L - k_P)$, P = phenylalanine concentration and L = leucine concentration The assumptions are

$$K_p = \frac{E_f P}{E_p}, K_1 = \frac{E_f L}{E_L}, e = E_f + E_L + E_p$$

and

$$v = ke = k_F E_f + k_L E_L + k_P(e - E_f - E_L)$$

Where E_f, E_L, and E_p represent free, leucine-bound and phenylalanine-bound enzyme, respectively, v is the velocity of thermal inactivation and e is total enzyme

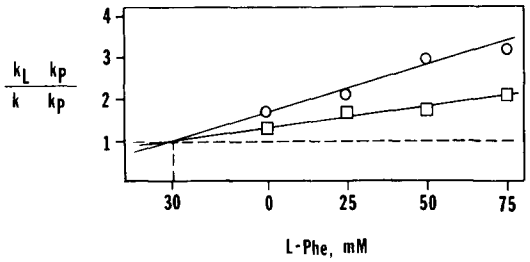

Fig 5 Dixon-type plot of thermal inactivation of aspartokinase in the presence of combinations of leucine and phenylalanine Thermal inactivation rates were obtained at 56°C as described in the text $(k_L - k_P)/(k - k_P)$ is plotted versus phenylalanine concentration at two fixed concentrations of leucine ○, 5 mM leucine, □, 10 mM leucine k_L and k_P are first-order rate constants for thermal inactivation at saturating concentrations of leucine and phenylalanine, respectively k is the observed rate constant with the various combinations of leucine and phenylalanine shown It can be shown that for mutually exclusive binding (competitive) the two lines intersect at a value of unity on the y axis and a value of $-K_P(k_F - k_L)/(k_L - k_P)$ on the x axis k_F is the first-order rate constant for thermal inactivation in the absence of amino acids K_D is the dissociation constant for the enzyme phenylalanine complex In this experiment, k_F = 1 14 min^{-1}, k_P = 0 56 min^{-1} and k_L = 0 15 min^{-1} K_D estimated from these data is 12 mM The least square lines are, y = (0 0214 ± 0 0037) x +1 7 ± 0 18 for 5 mM leucine and y = (0 00984 ± 0 0021) x 1 32 ± 0 098 for 10 mM leucine The intersection point is y = 1 04, x = −30 Aspartokinase specific activity was 18 units/mg

While kinetic experiments suggest that phenylalanine and leucine bind to the same site or to mutually exclusive sites on aspartokinase, it is clear that the conformations induced or stabilized by these ligands are grossly different Both are more stable to heat than the native conformations but their first-order rate constants for inactivation differ by about 5-fold Arrhenius activation energies also differ by about 31 kcal/mol Furthermore, in proteolysis experiments, the phenylalanine conformer is more labile, while the leucine conformer is more stable In view of their cooperative heterotropic interactions, it may be presumed that lysine and the hydrophobic amino acids bind at different sites However, in view of the apparent broad specificity of the hydrophobic site, there may be some lysine binding to it at high lysine concentrations There is evidence for this in the aspartokinase from another strain of *E coli* [9] The concerted model of Monod et al [8] has features which explain much of the behavior of various aspartokinases [9,20] When the effects of monovalent cations and hydrophobic amino acids are considered, one must postulate many spontaneously arising conformations in order to stay with the simple concerted model It is simpler to postulate that certain conformations are ligand induced, perhaps superimposed on a basic conformational equilibrium of the concerted type With the apparent binding of leucine and phenylalanine at the same site, the differences observed in the properties of the two conformers are most likely due to ligand-induced differences

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