PROTECTION OF THE PROLINE-AND VALINE-ACTIVATING ENZYMES BY THEIR AMINO ACID SUBSTRATES AGAINST THERMAL INACTIVATION[1]

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It is well-known that many enzymes are stabilized by the presence of their substrates. The increased stability is usually attributed to the highly specific and reversible interaction between the enzyme and the substrate to form an enzyme-substrate complex which is less readily inactivated than is the free enzyme. A protection constant (π), analogous to a Michaelis-Menten constant (Km), has been defined empirically (Burton, 1951) as the concentration of substrate which will provide half-maximal protection against spontaneous inactivation at a given pH and temperature. Kinetic analysis of the thermal inactivation of D-amino acid oxidase enabled Burton to conclude that the experimentally determined protection constant could be identified with the dissociation constant (α) for the enzyme-protector complex in the reversible reaction: enzyme + protector $\underset{k_2}{\overset{k_1}{\rightleftharpoons}}$ enzyme-protector complex, where $\pi = \alpha = \frac{k_2}{k_1}$. Thus, the kinetics of the thermal inactivation of the enzyme could be used to estimate the equilibrium constant in the binding of either a substrate, a cofactor or a competitive inhibitor (singly or in combination) in the absence of other reactants, and with no formation of product.

In view of the known instability of many of the amino acid activating enzymes, we considered it worthwhile to explore the possibility of using the thermal inactivation technique as an adjunct to conventional methods of enzyme kinetics to measure the biological binding affinity between amino acid

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activating enzymes and their substrates. In this work we have employed a crude preparation of the proline-activating enzyme and a purified preparation of the valine-activating enzyme. The protection constants for the amino acid substrates (proline and valine, respectively) have been determined in the absence of products (pyrophosphate, AMP, hydroxamate) of the activation reaction and in the absence of the additional reactants (ATP, sRNA, NH_2OH) that are usually present in the system when the kinetics of the enzymes are being studied. The results are encouraging. The kinetics can be interpreted in terms of a reversible formation of an enzyme-substrate complex, and protection constants have been determined which are in fair agreement with the respective Michaelis-Menten constants determined by conventional methods.[4]

Methods. An enzyme fraction enriched in proline-activating activity was prepared by homogenizing rat liver in 2.5 volumes of a buffered sucrose-salts solution (medium A) (Littlefield, et al., 1957) followed by centrifugation at 78,000xg for 3 hr. The proline-activating enzyme was then precipitated with 0.5 volume of ethanol at -10°. The precipitate was redissolved in medium A, and the solution was treated with protamine sulfate to remove nucleic acid followed by dialysis against 0.01 M Tris buffer, pH 7.7. The enzyme was used immediately after dialysis.

Purified E. coli valine-activating enzyme was prepared as described by Bergmann, et al. (Bergmann, et al., 1961). The enzyme was re-chromatographed two additional times on DEAE-cellulose columns to insure maximal removal of traces of the isoleucine-activating enzyme. The enzyme was stored in -15° in 0.02 M phosphate buffer, pH 7.5, containing 10^{-3} M reduced glutathione.

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4. It is to be noted that π and α are true dissociation constants and should be related more exactly to Ki (inhibition constant) or to Ks (substrate constant) rather than to Km (Michaelis-Menten constant), where $Ks = k_2/k_1$ and $Km = (k_2 + k_3)/k_1$ in the reaction $E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} Prod.$; and where $Ki = k_5/k_4$ in the reaction $E + I \underset{k_5}{\overset{k_4}{\rightleftharpoons}} EI$.

The thermal inactivation of the two enzymes was studied using 16x125 mm culture tubes in a thermostatically controlled water bath. Each tube contained in a total volume of 0.5 ml: enzyme, 100 μmoles Tris buffer, pH 8.0, and the compound to be tested for its stabilizing properties. In addition, 5 μmoles Mg^{+2} was included with the proline enzyme; however, no Mg^{+2} was included with the valine enzyme. After the period of heating was completed, the tubes were cooled in melted ice, and appropriate reagents were added, to a final volume of 1 ml, for the assay of residual enzyme activity at 37°. Proline activity was assayed by the colorimetric hydroxamate method (Atherly and Bell, 1964); valine activity was assayed by the ATP:PP^{32} exchange method (Bergmann, et al., 1961).[5]

Kinetics of thermal inactivation and stabilization by amino acid substrates. Conditions were found where the spontaneous inactivation of the proline- and valine-activating enzymes approximated at first-order course over a range of elevated temperatures. Figure 1a shows typical results obtained with the valine-activating enzyme. Figure 1b shows that the magnitude of the rate constants is related linearly to the inactivation temperature approximately as described by the Arrhenius equation. When the amino acid substrate was present during the thermal inactivation step, the first-order course of inactivation was retained; however, the value of the rate constant of inactivation was reduced in proportion to the concentration of the added substrate, indicating that the enzymes had been partially stabilized by the presence of their respective substrates. No reversal of the inactivation has been observed after the partially inactivated enzyme has been stored at 3°C in the presence of 10^{-3} M reduced glutathione for as long as 48 hours following the heating procedure.

5. In each series of tubes assayed for residual enzyme activity, appropriate amounts of either proline or valine were added so that the total concentration of substrate was 10^{-2} M in all tubes. In instances where another compound was employed as protector, appropriate controls were included to compensate for the effect of this compound on the activation of proline or valine.

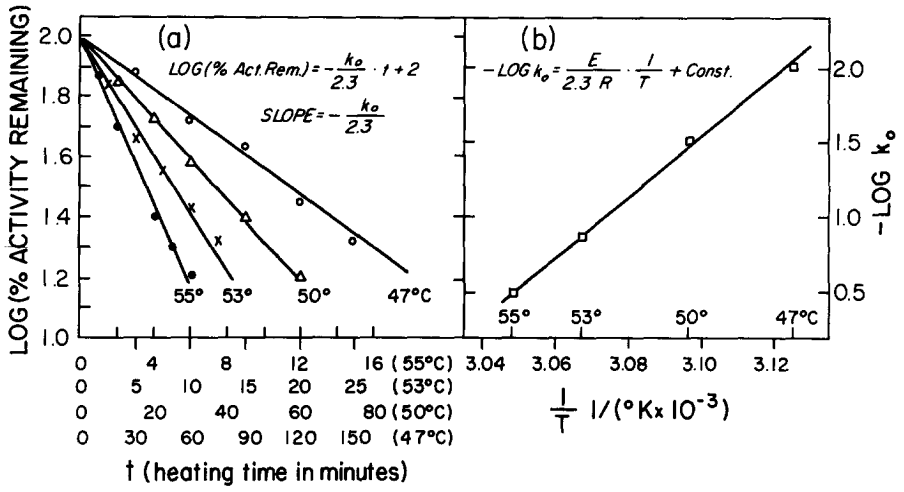

Fig. 1. E. coli valine-activating enzyme: (a) First-order course of thermal inactivation at different heating temperatures in the absence of substrate. k_o is the first-order rate constant of thermal inactivation in the absence of protecting substrate. (b) Arrhenius plot relating k_o to heating temperature. (Similar data have been obtained with the proline-activating enzyme.)

TABLE I. SPECIFICITY OF STABILIZATION BY AMINO ACIDS

Proline-Activating Enzyme		Valine-Activating Enzyme	
Protector	% Activity Remaining*	Protector	% Activity Remaining*
None	5 - 15	None	20 - 30
L-Proline (5)**	80 - 90	L-Valine (10)**	70 - 80
L-Azetidine-2-Carboxylic Acid (50)	80 - 90	L-α-Aminobutyric Acid (100)	50 - 60
L-Hydroxyproline (50)	5 - 15	Cycloleucine (140)	50 - 60
D,L-Pipecolic Acid (50)	5 - 15	L-Isoleucine (10)	25 - 35
D-Azetidine-2-Carboxylic Acid (50)	5 - 15	L-Leucine (10)	20 - 30
		D-Valine (10)	20 - 30

* The proline and valine enzymes were heated at 53° for 5 and 10 minutes, respectively, as described in Methods.

** The numbers in parentheses indicate the μmoles of protector per 0.5 ml of heated enzyme solution. The residual enzyme activity was assayed with either L-proline or L-valine (10^{-2} M) as described in Methods.

Specificity of the stabilization by amino acids. The specificity of the two enzymes for a particular group of amino acids capable of providing protection against thermal inactivation closely parallels the specificity of the two enzymes in the activation reaction. Thus, in addition to L-proline, the proline-activating enzyme was stabilized by high concentrations of L-azetidine-2-carboxylic acid which has been reported (Atherly and Bell, 1964) to be a substrate for the proline-activating enzyme (Table I). The valine-activating enzyme was stabilized by high concentrations of L-α-aminobutyric acid and cycloleucine in addition to L-valine; the former two compounds are known to be substrates for the valine-activating enzyme (Bergmann, et al., 1961; Lu and Bell, 1964). The D-isomers of the substrates tested did not stabilize either of the enzymes, nor did several compounds which are structurally similar to the natural substrate but do not effectively serve as substrates themselves. The results are summarized in Table I.

Comparison of π with Km at 37°. The Km of the two enzymes with their respective substrates was obtained at 37° where the enzymes are reasonably stable.

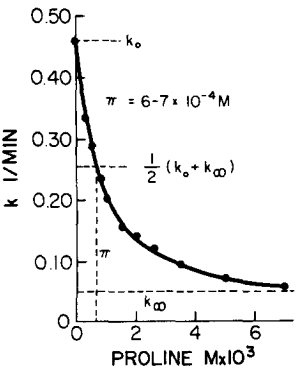

Fig. 2. Determination of π at 53° for proline with the proline-activating enzyme. The heating and assay system was as described in Methods. k_o and k_∞ are the first-order rate constants of inactivation in the absence of proline and in the presence of saturating concentrations of proline, respectively. It can be shown that the curve is described by the equation

$$k = (k_o\alpha + k_\infty P)/(\alpha + P)$$

which is derived assuming a reversible formation of an E-P complex. Under these conditions π is equivalent to α. An alternate and more sensitive graphic method for determining π involves the use of the above equation in a linearized form analogous to the Lineweaver-Burk and Eadié-Hofstee graphic methods used in enzyme kinetics to relate initial reaction velocity to substrate concentration.

The value of π at 37° was estimated by first determining values of π at four elevated temperatures and then extrapolating to 37° by means of a van't Hoff plot which defines the effect of temperature on equilibrium constants. To obtain a value for π at each elevated temperature, rate constants of inactivation (k) were determined over a range of substrate concentrations; the value of π at that given temperature was then determined from a plot of k against protector concentrations as illustrated in Figure 2. The van't Hoff plots for both enzymes are shown in Figure 3.

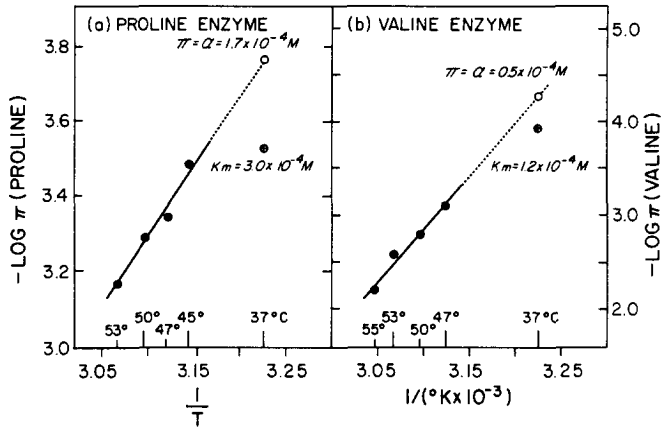

Fig. 3. Van't Hoff plot relating π to heating temperature for (a) proline-activating enzyme and (b) valine-activating enzyme. (●), π values determined experimentally at the indicated temperatures; (o), π at 37°C estimated by extrapolation; (●), Km at 37°.

The estimated values for π at 37° are in fair agreement with the Km values. With both enzymes, the π values are somewhat smaller than the respective Km values; this is as would be expected if the Michaelis constant (Km) is larger than the substrate constant (Ks).

Further work is in progress to evaluate this technique.

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