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Steady-State Kinetics of Aminopeptidase Catalysis: A Stopped-Flow Radiationless Energy Transfer Study

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Stopped-flow radiationless energy transfer experiments have been carried out to investigate the hydrolysis of some dansyl peptide substrates (S) catalyzed by aminopeptidase (E). RET between enzyme tryptophanyl residues and the dansyl group in the substrate allowed direct observation and quantitation of the enzyme-substrate (ES) complexes. Analysis of the stopped-flow RET traces gives k_{cat} = 1.32 s^{-1} and K_M = 47 μM for Leu-Ala-NH$(CH_2)_2$NH-Dns (Leu-Ala-DED) and k_{cat} = 4.80 s^{-1} and K_M = 196 μM for Leu-Gly-NH$(CH_2)_2$NH-Dns (Leu-Gly-DED). The activation energies of the enzymatic reactions were determined from the Arrhenius plots to be 57 and 38 kJ·mol^{-1} for Leu-Ala-DED and Leu-Gly-DED, respectively. The kinetic results indicate that the enzyme binds Leu-Ala-DED more tightly than Leu-Gly-DED as revealed by a small value of K_M. That this enzyme catalyzes the turnover of Leu-Gly-DED more efficiently than Leu-Ala-DED is reflected in a large value of k_{cat} and a small activation energy. The RET signals during the hydrolysis of Leu-Val-NH$(CH_2)_2$NH-Dns were extremely weak probably because of the inefficient energy transfer in the ES complex or the retention of the product in the enzyme after completion of the reaction. Aminopeptidase was inactive towards the dansyl compounds of the single amino acid studied. This fact may be due to an unfavorable conformation of these compounds in the ES complexes (small k_{cat}) or a weak binding of the substrates to the enzyme (large K_M) or both.

INTRODUCTION

Leucine aminopeptidase (EC 3.4.11.1) is an exopeptidase that catalyzes the hydrolysis of *N*-terminal amino acids of peptides and proteins.[1-3] Several aminopeptidases have been purified; their physical properties, specificities and metal ion requirements have been characterized to varying extents.[2-9] The action of this enzyme is not limited to leucine compounds; it hydrolyzes many peptides and amides that possess a free amino group and the L-configuration. Most aminopeptidases require metal ions for activity. The requirement of calcium ion by pronase aminopeptidase is exceptional. In all other cases, zinc,[10,11] cobalt,[12] magnesium[1,10] and manganese[1,10] are the required catalytic metal ions for enzymes in this family. However, little information about the kinetics of the hydrolysis of model substrates and the nature of the active site has been obtained because enzyme-substrate (ES) intermediates are both tansitory and present in small concentrations and because few techniques are available for the detection of these intermediates.

Radiationless energy transfer (RET) between enzyme tryptophanyl (Trp) residues as intrinsic donors and an extrinsically attached acceptor in the substrate allows direct observaation of the ES complexes under both steady-state and pre-steady-state conditions. This technique has been successfully applied to several proteases using fluorescent 5-dimethylaminonaphthalene-1-sulfonyl (dansyl or Dns) substrates as energy acceptors.[14-18] Thus, the RET approach is an attractive prospect for the study of enzyme kinetics. We have synthesized several dansyl peptides with the general formula Leu-X-NH$(CH_2)_2$NH-Dns (Leu-X-DED), in which X is an amino acid, to investigate the kinetics of aminopeptidase catalysis by the RET method. As an example, the reaction of aminopeptidase and Leu-Ala-DED is given by:

$$\text{Leu-Ala-DED} \xrightarrow{\text{Aminopeptidase}} \text{Leu} + \text{Ala-DED}$$

Aminopeptidase from pronase has a molar mass of 25,000 Daltons and contains a calcium ion at the active site for enzyme activity and stability.[13] RET experiments permit determination of the steady-state kinetic parameters, k_{cat} and K_M, which directly reflect the catalytic and binding abilities of the enzyme towards the substrates. Such information is essential for detailed understanding of the chemi-

Dedicated to Professor Sheng-Lieh Liu (劉盛烈) on the occasion of his eightieth birthday.

cal events occurring during catalysis.

EXPERIMENTAL SECTION

Materials

Pronase (Calbiochem), EDTA, Tris, Leu-*p*-nitroanilide (LPNA), ethylenediamine, di-*tert*-butyl carbonate and all amino acids (Sigma), *N*-hydroxysuccinimide, 1,3-dicyclohexyl carbodiimide (Aldrich), $CaCl_2$, cyanogen bromide, dichloromethane, 1,6-hexanediamine, ethylacetate, silica gel (E. Merck), Sephadex G-25, CM cellulose (Whatman CM-52), sepharose 4B (Pharmacia) were obtained commercially. The substrates, Leu-X-DED, in which X = Glycine, Alanine or Valine, and Y-$NH(CH_2)_2NH$-Dns (Y-DED), in which Y = Glycine, Alanine, Valine and Leucine, were synthesized from the coupling of Leu-X (or amino acids) and dansylethylenediamine as described previously.[19]

Purification of the Enzyme

The aminopeptidase was isolated from pronase through Sephadex G-25 (1.5 x 30 cm), CM-cellulose (1.5 x 15 cm) and 1,6-hexanediamine agarose (1 x 10 cm) columns at 4°C according to the procedure of K. D. Vosbeck et al.[13] The activity of the enzyme against L-Leucine-*p*-nitroanilide was determined by minitoring the absorbance change at 405 nm (ε = 9900 M^{-1}-cm^{-1}) on a UV-*vis* spectrophotometer (Hitachi U-3210).

Direct Observation of ES Complexes

Most enzyme contains Trp residues which emit near 330 nm upon excitation with 280-nm light (Fig. 1A). When the substrate (S) is bound to the enzyme (E) to form an ES Complex, efficient energy transfer from enzyme Trp residues to substrate dansyl groups leads to quenching of the Trp fluorescence and simultaneous generation of dansyl fluorescence (Fig. 1B). As the reaction is complete and the products leave the enzyme, the energy transfer terminates which results in disappearance of dansyl fluorescence and simultaneous recovery of Trp fluorescence (Fig. 1C). Hence, RET occurs only within the ES complexes. Changes in concentration of the ES-complex and quantum yields of Trp or Dns, result in changes in fluorescence, directly signaling the existence of the ES complex and allowing its quantitative determination. The degree of energy transfer is determined by the distance between, and the relative orientation of, the Trp donor and the dansyl acceptor as described by the Förster theory.[20] As the distance and orientation of these groups differ slightly in the individual intermediates, this technique provides a sensitive means to detect specific intermediates.

Stopped-Flow Experiments

The stopped-flow experiments were carried out on a stopped-flow accessory (SLM FP-052) of a spectrofluorometer (SLM-8000C) in conjunction with a low-temperature circulating bath (Firstek Scientific Model B204) for temperature control. Selective observation of Trp or Dns fluorescence was achieved by using a 355-nm bandpass filter or a 435-nm long-pass filter, respectively.

Determination of Steady-State Kinetic Parameters

An enzyme (E) catalyzes the conversion of substrate (S) to product (P) through a single intermediate, ES, as shown below:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \qquad (1)$$

Under steady-state conditions, the reaction rate (v) is given by the Michaelis-Menten equation:

$$v = -\frac{d[S]}{dt} = [E]_o[S]k_{cat}/(K_M + [S]) = k_{cat}[ES] \qquad (2)$$

in which $[E]_o$ is the total enzyme concentration, [S] and [ES] are the concentrations of substrate and complex, respectively. k_{cat} and K_M are related to the rate constants for the elementary steps by:

$$k_{cat} = k_2 \qquad (3)$$

$$K_M = (k_{-1} + k_2)/k_1 \qquad (4)$$

Fig. 2 shows the diagram of the RET traces illustrating the definition of experimental parameters used to

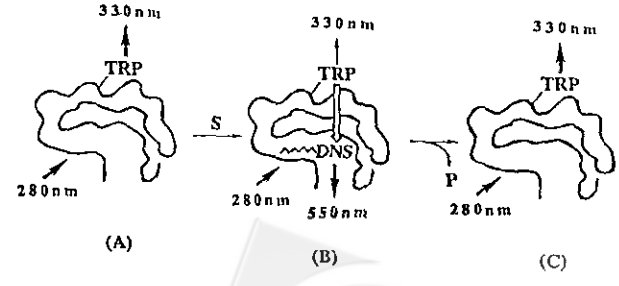


Fig. 1. Diagram of direct observation of ES complex by radiationless energy transfer during enzyme catalysis. Tryptophanyl and dansyl fluorescence are indicated by 330- and 550-nm emissions which occur only in the ES complex.

evaluate k_{cat} and K_M. F_t is the magnitude of the fluorescence change at period t after initiation of the reaction and F_o is F_t at $t = 0$. A_t (A_o) is the area between the abscissa and the RET trace from time t (0) to completion of the reaction. The area was estimated using the software of the SLM spectrofluorometer.

F_t is directly proportional to [ES] in a RET experiment and is given by:

$$F_t = C[ES] \tag{5}$$

in which C is a proportionality constant. Combining Eqs. 2 and 5 gives

$$F_t = -\frac{C}{k_{cat}}\frac{d[S]}{dt} \tag{6}$$

Integration of Eq. 6 leads to:

$$A_t = \int_t^\infty F_t\,dt = -\frac{C}{k_{cat}}\int_t^\infty d[S] = \frac{C}{k_{cat}}[S]_t \tag{7}$$

Substitution of Eqs. 5 and 7 into Eq. 2 gives:

$$v = k_{cat}[ES] = \frac{k_{cat}F_t}{C} = \frac{k_{cat}F_t[S]_t}{A_t k_{cat}} = \frac{[E]_o[S]_t k_{cat}}{k_M + [S]_t}$$

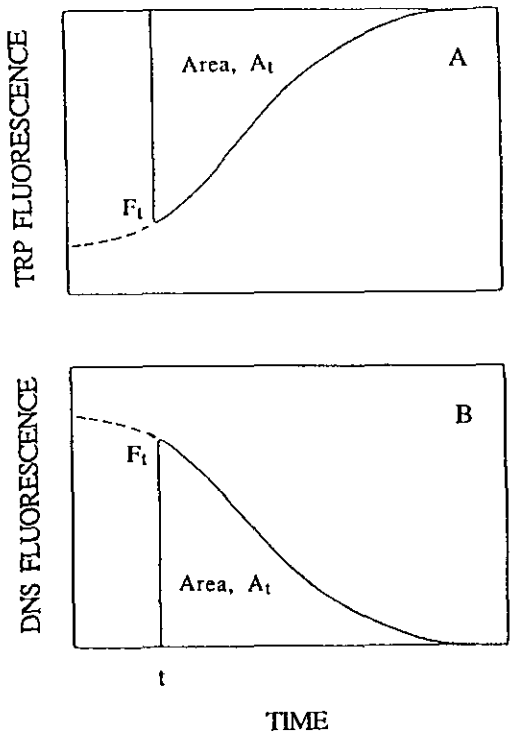


Fig. 2. Stopped-flow fluorescence traces showing the changes of (A) tryptophanyl and (B) dansyl fluorescence with time and defining the parameters F_t and A_t.

or in the double reciprocal form:

$$\frac{A_t[E]_o}{F_t[S]_t} = \frac{1}{k_{cat}} + \frac{K_M}{k_{cat}}\frac{1}{[S]_t} \tag{8}$$

The steady-state kinetic parameters, k_{cat} and K_M, were evaluated from the linear plot of $A_t[E]_o/(F_t[S]_t)$ vs $1/[S]_t$ at a single substrate concentration. The value of $[S]_t$ was calculated by using $[S]_t = (A_t/A_o)[S]_o$, which is a direct consequence of Eq. 7.

Eq. 8 is similar to the traditional Lineweaver-Burk plot ($[E]_o/v = 1/k_{cat} + (K_M/k_{cat})/[S]$) with the reaction rate replaced by $F_t[S]_t/A_t$. At $t = 0$, the measured value of $F_o[S]_o/A_o$ is equivalent to the initial rate of the reaction and Eq. 8 becomes:

$$\frac{A_o[E]_o}{F_o[S]_o} = \frac{1}{k_{cat}} + \frac{K_M}{k_{cat}}\frac{1}{[S]_o} \tag{9}$$

Therefore k_{cat} and K_M can also be evaluated from the linear plot of Eq. 9 from a series of stopped-flow traces obtained with different concentrations of substrate.

RESULTS AND DISCUSSION

Spectral Properties of the Enzyme and the Substrates

The absorption and fluorescence spectra of aminopeptidase and a representative dansyl substrate, Leu-Ala-DED, are shown in Fig. 3. The spectra of all other dansyl substrates are similar to those of Leu-Ala-DED. These substrates have the convenient property that the absorption spectrum of the dansyl group ($\lambda_{max} \sim 330$ nm) overlaps significantly with the emission spectrum of the enzyme Trp residues ($\lambda_{max} \sim 330$ nm) and the emission spectrum of the dansyl group ($\lambda_{max} \sim 550$ nm) is red-shifted far enough not to overlap with its own absorption spectrum and with the emission spectrum of the Trp residues. All these properties make this system an excellent energy donor-acceptor pair. Thus, when a dansyl substrate is bound to the enzyme and Trp is excited near its absorption maximum of 280 nm, the proximity of the two leads to efficient energy transfer which is manifested as dansyl fluorescence at 550 nm. The use of a 355-nm band-pass filter permited selective observation of the Trp emission and eliminated interference from the scattering of the excitation light and the emission from Dns fluorescence as shown in Fig. 3. Use of a 435-nm long-pass filter allows the selective detection of Dns fluorescence as shown in Fig. 3.

Determination of k_{cat} and K_M

Typical RET traces showing the changes in both Trp

and Dns fluorescence for the reaction of aminopeptidase (1.97 μM) and Leu-Ala-DED (123 μM) in Tris (5 mM), $CaCl_2$(5 mM), NaCl (0.5 M) at pH 8.0 and 25°C are shown in Fig. 4.

The sigmoidal shape of the stopped-flow traces is characteristic of those found in RET studies whenever $[S]_t > K_M$. Under these conditions, more than 50% of E is initially complexed with S to form the ES complex, the concentration and hence F_t of which decay slowly until $[S]_t$ approaches K_M. At this point, the ES decays more rapidly and eventually goes to zero as $[S]_t$ approaches zero.

Analysis of the RET traces for Trp and Dns fluorescence at a single concentration of substrate according to equation (8) gave straight lines as shown in Fig. 5. Using the linear least-squares fit, the values of k_{cat} and K_M were determined to be 1.34 ± 0.02 s^{-1} and 43 ± 1 μM for Trp fluorecence and 1.31 ± 0.04 s^{-1} and 46 ± 2 μM for Dns fluorescence; the results agree well.

The plots of $A_o[E]_o/(F_o[S]_o)$ vs $1/[S]_o$ for Trp and Dns fluorescence for the reaction of aminopeptidase and Leu-Ala-DED at various substrate concentrations in Tris (5 mM), $CaCl_2$ (5 mM) at pH 8.0 and 25°C are shown in Fig. 6. Both plots are linear and the values of k_{cat} and K_M were estimated by linear least-squares fits to be 1.32 ± 0.02 s^{-1} and

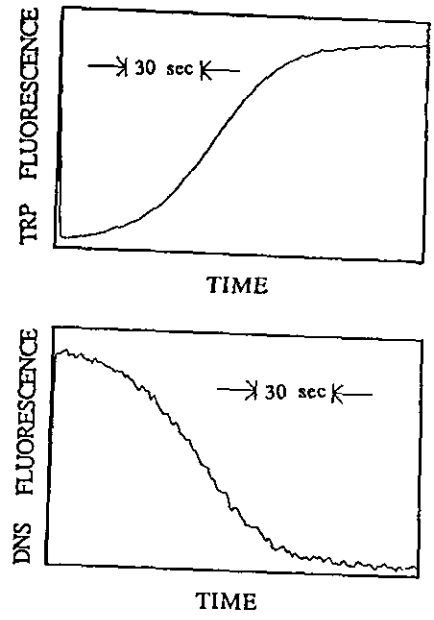


Fig. 4. Stopped-flow trace (λ_{ex} = 280 nm) showing the changes in Trp and dansyl fluorescence during the hydrolysis of Leu-Ala-NH$(CH_2)_2$NH-Dns (123 μM) catalyzed by aminopeptidase (1.97 μM) in Tris (5 mM), $CaCl_2$ (5 mM), NaCl (0.5 M) at pH 8.0 and 25° C.

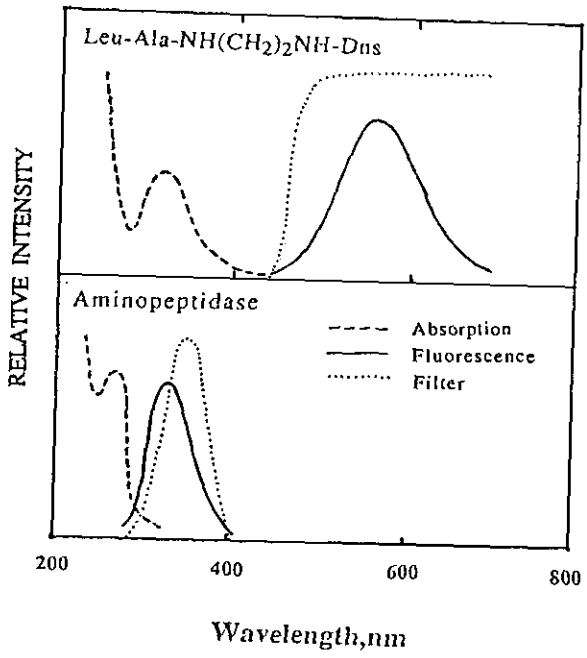


Fig. 3. Absorption and fluorescence spectra of aminopeptidase and Leu-Ala-NH$(CH_2)_2$NH-Dns showing spectral overlaps between enzyme emission and dansyl absorption. Appropriate filters enable monitoring selectively Trp and Dns Fluorescences; shown by the dotted curves.

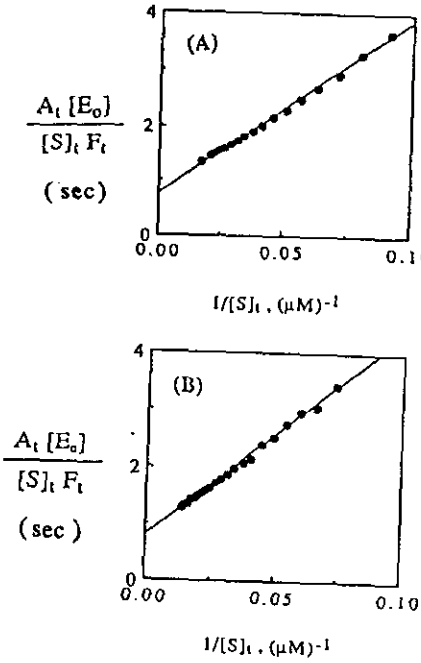


Fig. 5. Plots of $A_t[E]_o/(F_t[S]_t)$ versus $1/[S]_t$ for the hydrolysis of Leu-Ala-NH$(CH_2)_2$NH-Dns (123 μM) catalyzed by aminopeptidase (1.97 μM) in Tris (5 mM), $CaCl_2$(5 mM), NaCl(0.5 M) at pH 8.0 and 25°C. The kinetic parameters obtained are k_{cat} = 1.34 s^{-1} and K_M = 46 μM from Dns fluorescence.

49 ± 1 μM from Trp fluorescence and 1.30 ± 0.04 s^{-1} and 49 ± 2 μM from Dns fluorescence. The results also agree well with each other and with those obtained from the single RET experiments.

Similar RET experiments have been carried out for other dansyl substrates. The average values of k_{cat} and K_M were 4.80 ± 0.09 s^{-1} and 196 ± 4 μM for the hydrolysis of Leu-Gly-DED catalyzed by aminopeptidase under the same experimental conditions. The RET signals for the hydrolysis of Leu-Val-DED catalyzed by aminopeptidase were too weak for us to obtain reliable estimates of the kinetic parameters. Thin-layer chromatographic (TLC) analysis of the reaction mixture on silica plates in a solvent system consisting of chloroform: methanol: acetic acid (95 : 20 : 3) clearly indicated the complete disappearance of the fluorescent spot of Leu-Val-DED and the appearance of a distinct fluorescent spot due to Val-DED. The reason for lack of a significant RET signal is discussed hereafter. For the remaining dansyl substrates (Y-DED, in which Y = Gly, Ala, Val, Leu), there was no indication of hydrolysis from both stopped-flow and TLC experiments.

According to the Michaelis-Menten mechanism, [ES] = $[E]_o[S]/(K_M + [S])$ (Eq. 2). Solution for K_M gives $K_M = [E][S]/[ES]$, which is the dissociation constant of the ES complex under steady-state conditions. As $v = k_{cat}[ES]$, the activity of the enzyme is determined by its catalytic ability (given by k_{cat}) and by its substrate-binding affinity (given by K_M). Thus, the activity of the enzyme is commonly expressed as k_{cat}/K_M. The values of k_{cat} and K_M for the hydrolysis of Leu-Gly-DED catalyzed by aminopeptidase are all larger than those of Leu-Ala-DED. Therefore the enzyme binds Leu-Ala-DED more tightly (smaller K_M), and Leu-Ala-DED enters the active site of the enzyme much more easily, than Leu-Gly-DED. This fact may result from a favorable shape and size of the active site to accommodate the substrate and from the assistance of nearby amino-acid residues towards its binding. This enzyme seems to catalyze the turn over of Leu-Gly-DED in the ES complex more efficiently than Leu-Ala-DED (larger k_{cat}) which is consistent with the smaller activation energy of Leu-Gly-DED.

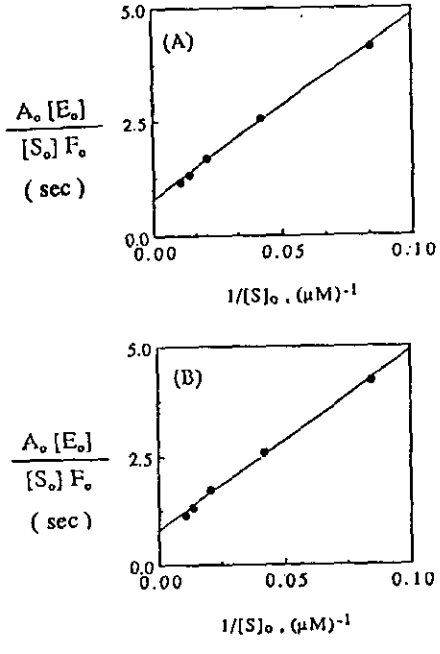


Fig. 6. Plots of $A_o[E]_o/(F_o[S]_o)$ vs $1/[S]_o$ for the reaction of aminopeptidase (1.97 μM) with various concentrations of Leu-Ala-NH$(CH_2)_2$NH-Dns in Tris (5 mM), $CaCl_2$ (5 mM), NaCl (0.5 M) at pH 8.0 and 25°C. The kinetic parameters were calculated to be k_{cat} = 1.32 s^{-1} and K_M = 49 μM from Trp fluorescence and k_{cat} = 1.29 s^{-1} and K_M = 49 μM from Dns fluorescence.

Determination of the Activation Energies

The stopped-flow experiments for the hydrolysis of Leu-Gly-DED and Leu-Ala-DED catalyzed by aminopeptidase in Tris (5 mM), $CaCl_2$ (5 mM), NaCl (0.5 M) at pH 8.0 have been carried out in the temperature range 5-30 °C. The substrates exhibited Michaelis-Menten kinetics over this temperature range because all the plots of Eq. 8 are linear. Their steady-state kinetic parameters were determined from the RET traces similarly to that described previously. The Arrhenius plots of Leu-Ala-DED and Leu-Gly-DED are linear over this temperature range indicating that the rate-determining step does not change under these conditions. The activation energies determined by linear least-squares fits were (57 ± 2) kJ·mol^{-1} and (38 ± 1) kJ·mol^{-1} for Leu-Ala-DED and Leu-Gly-DED, respectively.

The action of the enzyme is to provide a catalytic pathway with decreased activation energy. The special three-dimensional structure of the enzyme provides an active site for the reaction to occur; the nearby amino acid residues at the active site may also assist the reaction directly or indirectly, thereby diminishing the activation energy. Different substrates may experience different environments at the active site and the catalytic effects of the enzyme on various substrates may vary appreciably. That the activation energy for the hydrolysis of Leu-Gly-DED is 19 kJ·mol^{-1} smaller than that of Leu-Ala-DED indicates that aminopeptidase catalyzes the hydrolysis of Leu-Gly-DED more efficiently than Leu-Ala-DED.

Effects of Products on Aminopeptidase Catalysis

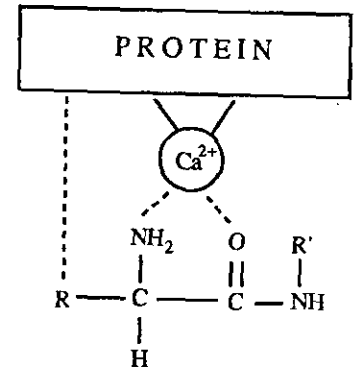
Determination of the steady-state kinetic parameters from the analysis of the stopped-flow RET traces is done under the assumption that the products do not inhibit the enzymatic reaction. We examined the effects of the products (Leu, Gly-DED, and Ala-DED) on catalysis by measuring the rate of hydrolysis of LPNA (1 mM) catalyzed by aminopeptidase in the presence of each product (0.3 mM, the maximum substrate concentration used in stopped-flow experiments). The rate was determined by following the absorbance change at 405 nm. The results indicate that neither product inhibits aminopeptidase catalysis appreciably (the rates are affected less than 2%). Hence these products do not compete significantly with LPNA for the active site of the enzyme. The value of K_M for LPNA determined from initial rate measurements (data not shown) is 0.29 mM which is larger than those of Leu-Gly-DED (0.196 mM) and Leu-Ala-DED (0.047 mM) indicating that the enzyme binds LPNA less tightly. Therefore inhibition of these products during the hydrolysis of these two dansyl substrates catalyzed by aminopeptidse was negligible and the obtained kinetic parameters are reliable. The extremely weak binding abilities (i.e. negligible inhibition) of Gly-DED and Ala-DED for aminopeptidase may also explain why the enzyme is incapable of hydrolyzing these two dansyl compounds.

Val-DED and Leu-DED inhibit significantly the hydrolysis of LPNA. The initial rates of hydrolysis of LPNA (1mM) are decreased by 45% and 40% by Val-DED and Leu-DED (0.3 mM), respectively. This result implies that the enzyme binds tightly Val-DED and Leu-DED. TLC results show no indication of hydrolysis for Val-DED and Leu-DED. All dansyl compounds of the single amino acid studied are not hydrolyzed by aminopeptidase probably because of an unfavorable conformation for catalysis in the ES complex (small k_{cat}) or weak binding of the substrate to the enzyme (large K_M) or both.

According to the Förster theory, the degree of energy transfer depends on the distance and orientation of the donor-acceptor pair (no energy transfer for perpendicular electric dipoles of the donor and acceptor). Different substrates may experience different environments in the active site, so to result in energy transfer to different degrees and hence in different signal intensities in the RET experiments for various substrates. In the case of Leu-Val-DED, there is an additional factor to be considered. As the enzyme binds Val-DED tightly, the product may not leave the enzyme after completion of the reaction. Therefore energy transfer may also occur in the enzyme-product complex which would make the observed RET signal much weaker.

According to the suggestions of Smith,[21] the role of metal ion in aminopeptidase (as in other metallopeptidases) is to act as a bridge during the formation of a coordination complex between the enzyme and the substrate. The sites of attack by the metal ion are believed to be at the free amino group and at the peptide carbonyl oxygen of the substrate (see Scheme I).[22] In addition, there is a hydrophobic interaction between the R group and similar groups on the enzyme.

Scheme I



Although this three-point model is only tentative and may subject to modification as further evidence is forthcoming, it is still a useful hypothesis to describe the variation of enzyme activities. Substrates with the three sites oriented in proper positions bind the enzyme tightly and are possibly hydrolyzed readily as well. The fact that Leu-DED and Val-DED strongly inhibit the hydrolysis of LPNA is consistent with strong hydrophobic interactions between the large aliphatic groups (e.g. Leu and Val) and the enzyme. For substrates with small aliphatic groups (e.g. Gly and Ala), the hydrophobic interactions are extremely weak; hence their inhibitory abilities are negligible.

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Key Words

Stopped-flow radiationless energy transfer; Hydrolysis kinetics; Aminopeptidase; Dansyl substrates.

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