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Kinetic Study of *S. griseus* Aminopeptidase by Stopped-flow Fluorescence Energy Transfer

Hsien-Cheng Yu (游憲政) and Wann-Yin Lin* (林萬寅)
Department of Chemistry, National Taiwan University, Taipei, Taiwan 10764, R.O.C.

The stopped-flow fluorescence traces for the hydrolysis of Leu-Ala-DED catalyzed by *Streptomyces griseus* aminopeptidase isolated from pronase exhibits a two-phase fluorescence change with comparable rates of formation and breakdown of the ES complex. We have developed a method for the determination of the individual rate constants from the stopped-flow traces. The kinetic parameters for the enzyme-catalyzed hydrolysis of Leu-Ala-DED at 25 °C and pH 8.0 are: $k_1 = (1.6 \pm 0.1) \times 10^5$ $M^{-1}s^{-1}$, $k_{-1} = 0.089 \pm 0.001$ s^{-1}, and $k_2 = 0.58 \pm 0.01$ s^{-1}. For the observed stopped-flow traces, the steady state approximation is valid only within a very small region around the maximal ES concentration and a large proportion (> 20%) of the substrate has already been hydrolyzed during the pre-steady state. For a very fast formation of ES complex, the steady state approximation is valid for almost the entire trace. The activation energies for each individual rate constant were determined to be 10.0 ± 0.7, 35 ± 5, and 21.2 ± 0.8 kcal mol^{-1} for k_1, k_{-1}, and k_2, respectively. Binding of E and S to form ES was accompanied by a decrease in Gibbs free energy, whereas a dramatic increase in free energy was observed for the conversion of ES to ES*. The dissociation of ES to form E and S had a very large activation energy, but it was also accompanied by a large increase in entropy.

INTRODUCTION

Radiationless energy transfer (RET) between the tryptophan (Trp) residue of the enzyme and the dansyl (5-dimethylaminonaphthalene-1-sulfonyl, Dns) group of the substrate is a very powerful probe for the direct observation of the ES complexes during the enzyme catalysis.[1-3] This technique has been successfully applied to several enzyme systems to study the steady state, pre-steady state, and inhibition kinetics as well as for distance measurements.[4-7] In most cases, the pre-steady state usually proceeds at a much faster rate than the steady state, and treatment of the pre-steady state kinetics is normally performed under the assumption of negligible turnover of the substrate.[7] In this paper, we have observed a two-phase stopped-flow RET trace with comparable rates of formation and breakdown of the ES complex during the hydrolysis of a dansyl substrate catalyzed by the *Streptomyces griseus* aminopeptidase (*S. griseus* AP) isolated from pronase. *S. griseus* AP is a calcium-activated monomeric enzyme with high enzyme activity and high thermal stability.[8,9] It prefers the substrates with a large, hydrophobic side chain (e.g., Leu) in the *N*-terminus. The primary and crystal structures of *S. griseus* AP have recently been determined.[10,11] The observed stopped-flow RET traces has revealed the hydrolysis of an appreciable amount of the substrate (> 20%) during the attainment of the maximal [ES] preventing the utilization of the conventional strategy for the treatment of the pre-steady state kinetics. In this paper, we have developed a method for determining the individual rate constants from the stopped-flow RET traces and studied the temperature dependence of these constants. We have also discussed the criteria for the validity of the steady state approximation and described a method to detect the interfering process $E + P \rightarrow ES$ from inspection of the stopped-flow RET traces.

EXPERIMETAL SECTION

Materials

S. griseus AP was isolated from pronase E (Merck Co., Germany) as described previously.[8] The substrate, L-leucyl-L-alanyl-dansylethylenediamine (Leu-Ala-DED), was prepared by the known procedure.[12] Tris, $CaCl_2$, and NaCl were obtained from Sigma Co., USA. Buffers and substrates were prepared in water purified to a specific resistance of 18 MΩ/cm by a Milli-Q system (Millipore).

Stopped-Flow Measurements

The stopped-flow experiments were performed on a Hitech SF-61 stopped-flow instrument (Hitech Scientific, UK) equipped with a sample handling unit (SHU-61), a dual

Dedicated to Professor Hsien-Ju Tien on the occasion of his 65th birthday.

detection accessory (OPT-680), a photodiode array assembly (MG-6010), a 75 W xenon lamp, and a circulating water bath for temperature control. A dual detection accessory allows the simultaneous observation of Trp (using a 320-nm band-pass filter) and Dns (using a 460-nm long-pass filter) fluorescence. All the experiments were carried out at 25°C in 10 mM Hepes, 5 mM $CaCl_2$, 0.1 M NaCl, pH 8.0.

Method for Determining the Individual Rate Constants

The stopped-flow RET traces shown in Fig. 1 can be described by the following mechanism:

$$E + S \underset{k_{-1}}{\overset{k_1}{\longleftrightarrow}} ES \xrightarrow{k_2} E + P \tag{1}$$

The changes in concentrations for the substrate ([S]) and the ES complex ([ES]) as a function of time are given by:

$$d[S]/dt = -k_1[E][S] + k_{-1}[ES] \tag{2}$$

$$d[ES]/dt = k_1[E][S] - (k_{-1} + k_2)[ES] \tag{3}$$

At the time of maximal [ES], t_m, the steady-state approximation ($d[ES]/dt = 0$) is valid. Thus

$$[ES]_m = [E]_m[S]_m/K_M \tag{4}$$

where $K_M = (k_{-1} + k_2)/k_1$ and the subscripts in $[E]_m$, $[S]_m$ and $[ES]_m$ denote the corresponding concentrations at t_m. $[ES]_m$ can also be calculated from the Michaelis-Menten equation:

$$[ES]_m = [E]_0[S]_m/(K_M + [S]_m) \tag{5}$$

where $[E]_0$ is the total concentration of the enzyme. The intensity of the stopped-flow RET trace, F_t, is directly proportional to [ES], thus

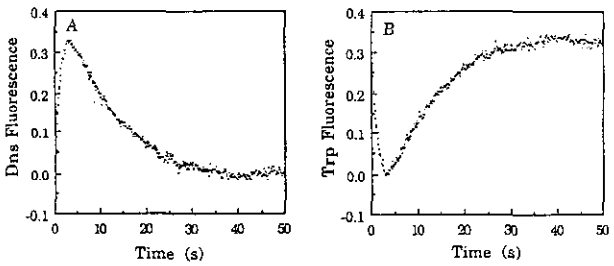

Fig. 1. Stopped-flow RET traces showing the changes in (A) Dns and (B) Trp fluorescence for the hydrolysis of 5.0 μM Leu-Ala-DED by 1.0 μM *S. gireus* AP in 10 mM Hepes, 0.1 M NaCl, 5 mM $CaCl_2$, at pH 8.0 and 25 °C.

$$F_t = c[ES] \tag{6}$$

where c is a proportionality constant. According to mechanism (1), the concentration of the product at time t, $[P]_t$, is given by:

$$[P]_t = \int_0^t k_2[ES]dt = (k_2/c)\int_0^t F_t dt = (k_2/c)A_t \tag{7}$$

where $A_t = \int_0^t F_t dt$. At $t = \infty$, the reaction is complete and

$$[P]_\infty = [S]_0 = (k_2/c)A_\infty \tag{8}$$

where $A_\infty = \int_0^\infty F_t dt$. Since $F_m = c[ES]_m$, combining this relation with equations (5) and (8) gives:

$$A_\infty[E]_0/(F_m[S]_0) = 1/k_2 + (K_M/k_2)(1/[S]_m) \tag{9}$$

where $[S]_m = [S]_0 - [P]_m - [ES]_m = [S]_0 - (k_2/c)A_m - F_m/c$. Since $c = k_2A_\infty/[S]_0$ (from equation (8)), $[S]_m$ can be expressed as:

$$[S]_m = ([S]_0/A_\infty)(A_x - F_m/k_2) \tag{10}$$

where $A_x = A_\infty - A_m = \int_m^\infty F_t dt$. Equations (9) and (10) were employed to determine the values of k_2 and K_M from a family of stopped-flow RET traces at various substrate concentrations using an iterative procedure: A value of $[S]_m$ was evaluated from equation (10) by neglecting the term F_m/k_2 (i.e. $[ES]_m$) and a double reciprocal plot of equation (9) gave the first estimate of k_2 and K_M. The obtained value of k_2 was then used in equation (10) to calculate $[S]_m$, and a similar double reciprocal plot gave a second estimate of k_2 and K_M. The iteration procedure continued until consistent values of k_2 and K_M were obtained.

At $t = 0$, equation (3) can be written as $(d[ES]/dt)_0 = (dF_t/dt)_0/c = R_0/(k_2A_\infty/[S]_0) = k_1[E]_0[S]_0$ or

$$R_0/A_\infty = k_1k_2[E]_0 \tag{11}$$

where $R_0 = (dF_t/dt)_0$, the initial slope of the stopped-flow RET trace. Therefore, measurement of R_0 and A_∞ at several enzyme concentrations allowed the evaluation of k_1k_2 from the linear plot of equation (11). The individual rate constants k_1, k_{-1} and k_2 can finally be determined from the values of k_1k_2, k_2 and K_M. Using this method, the experiments need not be carried out under the condition of either $[S]_0 >> [E]_0$ or $[E]_0 >> [S]_0$, which are normally adopted for most of the kinetic analysis.

RESULTS AND DISCUSSION

Determination of the Individual Rate Constants

Typical stopped-flow RET traces showing the two-phase change in Trp or Dns fluorescence for the hydrolysis of Leu-Ala-DED catalyzed by *S. griseus* AP are given in Fig. 1. When the enzyme combined with the substrate and its Trp residues are excited at 280 nm, the proximity of Trp and Dns in the ES complex leads to an efficient energy transfer which is manifested as a decrease in Trp fluorescence and a concomitant increase in Dns emission. The amount of fluorescence (Trp or Dns) change is directly proportional to [ES]. Thus the fluorescence change (Trp or Dns) in the fast phase of Fig. 1 indicates the gradual formation of the ES complex. After reaching a maximum, the amount of ES complex begins to decrease as the reaction proceeds due to the turnover of the substrate. This is revealed by a decrease in Dns fluorescence and a corresponding recovery of Trp emission as illustrated by the slow phase in Fig. 1.

The stopped-flow RET traces obtained at various substrate concentrations were analyzed by the iterative procedure using equations (9) and (10) as described previously. Only a few iterations (~ 4) were needed to achieve consistent kinetic constants. All kinetic parameters were obtained from the average of five measurements. The linear plot of equation (9) is shown in Fig. 2, and the obtained values of k_2 and K_M are 0.58 ± 0.01 s^{-1} and 4.2 ± 0.1 μM, respectively. The enzyme binds Leu-Ala-DED more tightly than L-leucine-*p*-nitroanilide (LPNA). However, the enzyme activity (k_2/K_M) against Leu-Ala-DED is smaller than that of LPNA by almost an order of magnitude.[9]

The stopped-flow experiments have also been carried out at various enzyme concentrations using the dual-time acquisition mode of the instrument. The fast time-mode (t = 0 to t_m) was used to evaluate the initial slope (R_0) by an exponential fitting, whereas the entire trace was used to calculate the area under the curve (A_∞). The plot of R_0/A_∞ vs. $[E]_0$ is shown in Fig. 3, and the value of k_1k_2 is estimated to be $(9.2 \pm 0.1) \times 10^4$ $M^{-1}s^{-2}$. The individual rate constants are finally determined: $k_1 = (1.6 \pm 0.1) \times 10^5$ $M^{-1}s^{-1}$, $k_{-1} = 0.089 \pm 0.001$ s^{-1}, $k_2 = 0.58 \pm 0.01$ s^{-1}.

The values of k_1, k_{-1} and k_2 can also be determined from the fitting of a single stopped-flow RET trace using the Matlab software (The Math Works Inc., USA). It first solves the differential equations (2) and (3) for [S] and [ES] with a given set of rate constants (k_1, k_{-1} and k_2) by the Runge-Kutta method[12] and then starts the nonlinear regression using the three rate constants as adjustable parameters. During the regression, the proportionality constant c is estimated by $k_2A_\infty/[S]_0$. The result is demonstrated in Fig. 4 and the obtained rate constants, $k_1 = 1.4 \times 10^5$ $M^{-1}s^{-1}$, $k_{-1} = 0.098$ s^{-1} and $k_2 = 0.57$ s^{-1}, are in excellent agreement with those obtained from equations (9) and (11). The excellent agreement of the generated [ES] and the experimental data (Fig. 4) indicates the validity of the RET model (i.e., $F_t = c[ES]$).

In analyzing the stopped-flow RET traces, possible interference by the direct excitation of the substrate must be taken into account to obtain meaningful kinetic results.[14] Since the amount of fluorescence change in RET or direct excitation is strongly dependent on the types of dansyl substrates used,[15] it is necessary to establish the origin of the observed fluorescence change. In this paper, the contribution from the direct excitation of the substrate is negligible because there is essentially no change in dansyl fluores-

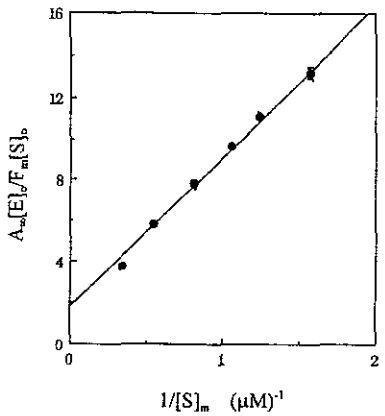

Fig. 2. The plot of $A_\infty[E]_0/F_m[S]_0$ vs. $1/[S]_m$ (from Dns fluorescence) for the hydrolysis of various concentrations of Leu-Ala-DED by 1.0 μM *S. gireus* AP in 10 mM Hepes, 0.1 M NaCl, 5 mM $CaCl_2$, at pH 8.0 and 25 °C.

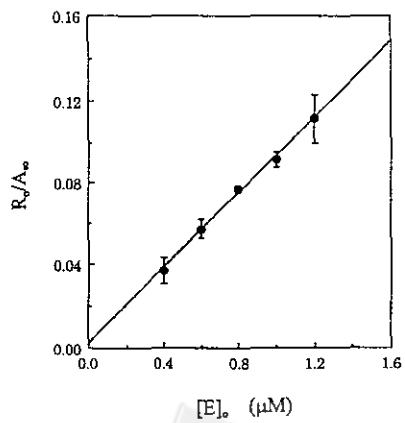

Fig. 3. The plot of R_0/A_∞ vs. $[E]_0$ (from Dns fluorescence) for the hydrolysis of 1.8 μM Leu-Ala-DED by various concentrations of *S. gireus* AP in 10 mM Hepes, 0.1 M NaCl, 5 mM $CaCl_2$, at pH 8.0 and 25 °C.

cence during the hydrolysis when the system is excited at 330 nm. Possible interference by the process E + P → ES is also unimportant because the TLC results show the total disappearance of the substrate after the reaction is complete. The presence of the process E + P → ES (with a rate constant k_{-2}) can also be examined by inspecting the stopped-flow RET traces. Fig. 5 show the traces calculated from the previously obtained rate constants (k_1, k_{-1} and k_2) and various values of k_{-2}. A non-zero RET intensity (i.e. non-zero [ES]) after the equilibrium has been established indicates the non-negligible process of E + P → ES.

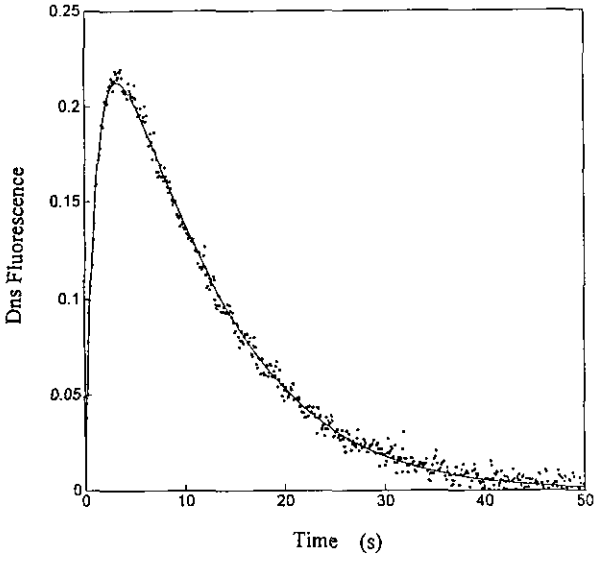

Fig. 4. Simulation of the stopped-flow RET trace for the hydrolysis of 1.8 μM Leu-Ala-DED by 1.0 μM *S. gireus* AP. Dots: experimental data; solid line: fitted curve with $k_1 = 1.4 \times 10^5$ $M^{-1}s^{-1}$, $k_{-1} = 0.098$ s^{-1} and $k_2 = 0.57$ s^{-1}.

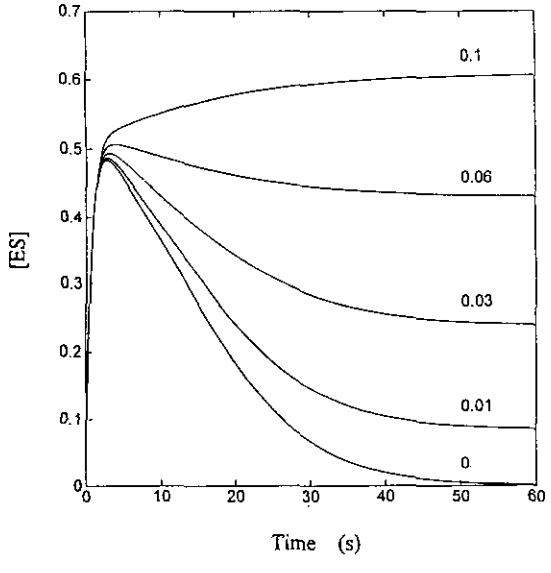

Fig. 5. The stopped-flow RET traces for the hydrolysis of 5 μM substrate by 1 μM enzyme calculated with $k_1 = 1.6 \times 10^5$ $M^{-1}s^{-1}$, $k_{-1} = 0.089$ s^{-1} and $k_2 = 0.58$ s^{-1}. The values of k_{-2} (in s^{-1}) were indicated in each trace.

Criteria for Validity of the Steady-State Approximation

Analysis of a stopped-flow RET trace for a single substrate concentration has previously been used to determine the kinetic parameters k_{cat} and K_M of enzyme-catalyzed reaction, provided that the steady state approximation is valid for a certain period of time and that $[S]_0 > K_M$.[2,7] To check the validity of the steady state approximation, we have calculated the value of $\delta = (d[ES]/dt)/(k_1[E][S])$, which is a measure of the deviation from steady state,[16] as a function of time by the Matlab software. Fig. 6A shows the time courses of $[ES]/[E]_0$, $[S]/[S]_0$ and δ for the hydrolysis of 5 μM substrate by 1 μM enzyme using the rate constants (k_1, k_{-1} and k_2) obtained in the previous section. The steady state approximation is valid only within a small region around t_m. Deviation greater than 20% is evident in the late stage of the reaction. At t_m, 22% of the substrate has already been hydrolyzed. For a fast formation of ES complex, the time courses of $[ES]/[E]_0$, $[S]/[S]_0$ and δ, calculated from the rate constants with a much larger k_1 (e.g., 10-fold) but keeping k_2 and K_M fixed, are shown in Fig. 6B. The steady-state approximation is valid after t_m (δ < 2% from t_m to 60 s). At t_m, 12% of the substrate has been hydrolyzed. Therefore, care

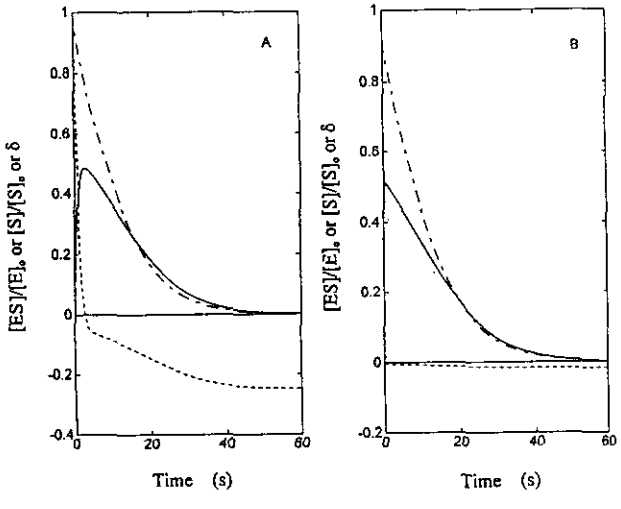

Fig. 6. Time courses of $[ES]/[E]_0$ (solid line), $[S]/[S]_0$ (dashed-dot line) and δ (dashed line) for the hydrolysis of 5 μM substrate by 1 μM enzyme. The curves were calculated by the Matlab software with (A) $k_1 = 1.6 \times 10^5$ $M^{-1}s^{-1}$, $k_{-1} = 0.089$ s^{-1}, $k_2 = 0.58$ s^{-1} and (B) $k_1 = 1.6 \times 10^6$ $M^{-1}s^{-1}$, $k_{-1} = 6.14$ s^{-1}, $k_2 = 0.58$ s^{-1}.

must be taken in using the steady state approximation and the assumption of non-turnover in the pre-steady state.

Temperature Dependence of the Individual Rate Constants

The stopped-flow experiments have also been carried out at various temperatures, and the data were analyzed by the same method described above to determine the thermodynamic and activation parameters for each individual step. The Arrhenius plots for each of the individual rate constants are shown in Fig. 7. All the plots are linear, and the slopes yield the activation energies (E_a) for the three rate constants k_1, k_{-1} and k_2. The results are listed in Table 1. The activation parameters associated with these rate constants can be calculated by the following relations: $\Delta G^{\neq} = RT \ln(k_B T/kh)$, $\Delta H^{\neq} = E_a - RT$, $\Delta S^{\neq} = (\Delta H^{\neq} - \Delta G^{\neq})/T$, where k_B, h and R are the Boltzmann, Planck, and gas constants, T the absolute temperature, and $k = k_1$, k_{-1} or k_2. Alternately, the values of $\Delta H^{\neq}$ and $\Delta S^{\neq}$ can also be obtained from the linear plot of $\ln(k/T)$ against 1/T according the the equation: $\ln(kh/k_B T) = \Delta S^{\neq}/R - \Delta H^{\neq}/RT$. The results are all listed in Table 1. The

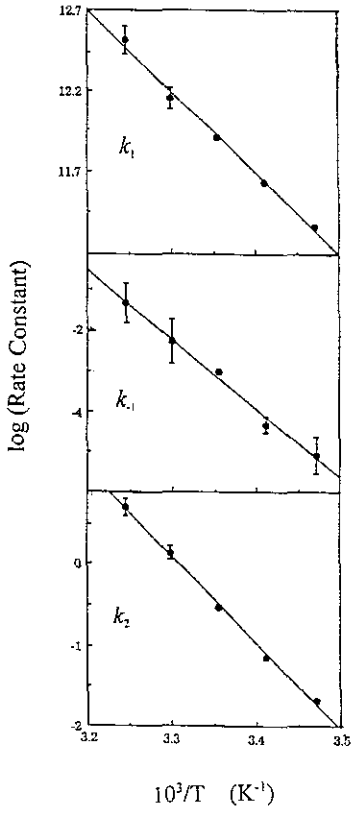

Fig. 7. The Arrhenius plots of the rate constants k_1, k_{-1} and k_2. The activation energies corresponding to the above rate constants were calculated from the slopes of the linear plots.

Table 1. Thermodynamic and Activation Parameters

Parameter	E_a (kcal mol^{-1})	$\Delta H^{\neq}$ or ΔH (kcal mol^{-1})	$\Delta S^{\neq}$ or ΔS (cal mol^{-1} K^{-1})	$\Delta G^{\neq}$ or ΔG^a (kcal mol^{-1})
k_1	10.0 ± 0.7	9.4 ± 0.6	-3.5 ± 0.3	10.4 ± 0.1
k_{-1}	35 ± 5	34 ± 5	50 ± 20	19.5 ± 0.5
k_2	21.2 ± 0.8	20.6 ± 0.8	9.4 ± 0.6	17.8 ± 0.1
K_M	-	-12.5 ± 0.7	-17 ± 2	-7.3 ± 0.2

[a] $\Delta G^{\neq}$ and ΔG were calculated at 25 °C.

effect of a large activation energy (35 kcal mol^{-1}) in the step ES → E + S (corresponding to k_{-1}) is somewhat compensated for by a large positive entropy of activation (50 cal mol^{-1} K^{-1}). As a result, the rate of dissociation of ES to form E and S is only about an order of magnitude smaller than that of the conversion of ES to ES*. The simultaneous observation of the three individual steps definitely depends on their relative rates. For a very fast pre-steady state (e.g., Fig. 6B), the free energy of activation for k_1 and k_{-1} must be much smaller than those listed in Table 1 if the turnover rates (e.g., k_2) are similar. The plot of $\ln(1/K_M)$ versus 1/T is also linear (data not shown), which gives the thermodynamic parameters ΔH, ΔS and ΔG (Table 1) for the process E + S → ES. A negative value of ΔG (-7.3 kcal mol^{-1} at 25 °C) indicates the favorable binding of E and S to form ES probably because of the complementary structures of the substrate and the active site of the enzyme.

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Key Words

Kinetic study; *S. griseus* aminopeptidase; Flouresence energy transfer.

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