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Stopped-Flow Cryoenzymological Investigation of the Pre-Steady-State Kinetics of Hydrolysis of Leu-Gly-NHNH-Dns by Leucine Aminopeptidase†

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Received September 9, 1987; Revised Manuscript Received January 25, 1988

ABSTRACT: Stopped-flow fluorescence experiments have been carried out to study the steady-state kinetics of hydrolysis of Leu-Gly-NHNH-Dns [Dns = 5-(dimethylamino)naphthalene-1-sulfonyl] by porcine kidney cytosol leucine aminopeptidase (LAP) in 50% v/v methanol/buffer solution at ambient temperature and the pre-steady-state kinetics of this reaction in the −35 to 0 °C temperature range. Experiments have been carried out on LAP species containing Mg(II), Mn(II), Cu(II), Ni(II), Zn(II), and no metal ion at the regulatory metal binding site. At ambient temperatures, the stopped-flow fluorescence changes observed on hydrolysis of the substrate have been used to measure the steady-state kinetic parameters k_{cat} and K_M. The results show that 50% v/v methanol lowers the values of k_{cat} from 2- to 12-fold compared to the reactions in the absence of methanol for all of the metallo-LAP, but that the values of K_M are essentially unaffected. The pre-steady-state reactions carried out under nonturnover conditions at −35 °C reveal a new relaxation for LAP species with Ni(II), Cu(II), and Zn(II) in the regulatory site. The value of k_{obsd} for this relaxation reaches a plateau at high substrate concentrations, and the magnitude of its fluorescence change at a fixed concentration of substrate is proportional to the enzyme concentration. Thus, this relaxation corresponds to the production and decay of a new enzyme–substrate intermediate not observed at higher temperatures whose fluorescence differs from that of the succeeding intermediate that is normally seen above −26 °C.

In the second of three papers in this issue (Lin et al., 1988), the stopped-flow fluorescence technique was used to study the steady-state kinetics of hydrolysis of Leu-Gly-NHNH-Dns by several metallohybrids of porcine kidney cytosol leucine aminopeptidase (LAP)[1] (EC 3.4.11.1) at 23 °C. In order to obtain more detailed information about the catalytic pathway for this reaction, these studies have been extended here to examine the pre-steady-state regime. More specifically, it is the goal of these studies to resolve elementary steps in the catalytic pathway with emphasis on the detection of new enzyme (E)–substrate (S) intermediates, ES_i. The stopped-flow fluorescence technique has been used successfully by others to observe reaction intermediates with fluorescent peptide substrates by utilizing either direct excitation of the S in ES_i (Sachdev & Fruton, 1975; Mattis & Fruton, 1976; Morgan & Fruton, 1978) or excitation of E in the ES_i, followed by resonance energy transfer to S (Latt et al., 1970; Auld et al., 1972; Auld & Holmquist, 1974; Auld, 1977; Lobb & Auld, 1979, 1980; Galdes et al., 1983, 1986; Auld & Prescott, 1983; Williams & Auld, 1986). On the basis of the results described in the preceding papers (Lin & Van Wart, 1988a; Lin et al., 1988), the direct excitation approach has been used in this study.

While stopped-flow fluorescence studies at ambient temperatures are capable of detecting ES_i, their resolving power is limited by the mixing time (>1 ms) of stopped-flow instruments. Hence, species that have lifetimes shorter than the mixing time will always escape detection. More rapid relaxation methods that depend on perturbations from equilibrium are unfortunately not well suited for the study of essentially irreversible reactions such as peptide hydrolysis. The resolving power of the stopped-flow technique, however, can be effectively increased if the reactions under study are carried out at lower temperatures, since the rates of interconversion of all intermediates are slowed in accordance with the Arrhenius equation. This effect of low temperature is the basis for

† Supported by National Institutes of Health Grant GM27276 and Research Career Development Award AM01066 to H.E.V.W. and National Science Foundation Grant DMB8520068.
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[1] Abbreviations: LAP, leucine aminopeptidase; $[(LAP)Zn_6M_6]$, metalloleucine aminopeptidase where (LAP) represents the hexameric apoenzyme and the brackets indicate the firm binding of six atoms each of Zn(II) and M(II) at the catalytic and regulatory sites, respectively; $[(LAP)Zn_6—]$, leucine aminopeptidase with Zn(II) at the catalytic site of each subunit and the six regulatory sites unoccupied; Tris, tris(hydroxymethyl)aminomethane; dansyl or Dns, 5-(dimethylamino)-naphthalene-1-sulfonyl; pH*, apparent protonic activity; E, enzyme; S, substrate; P, product; ES, enzyme–substrate intermediate.

0006-2960/88/0427-5068$01.50/0

cryoenzymology, the study of enzymatic reactions at subzero temperatures in cryosolvents (Fink & Greeves, 1979). Stopped-flow cryoenzymology is, therefore, a powerful tool for the study of the pre-steady-state kinetics of enzymatic reactions (Auld, 1977; Galdes et al., 1983, 1986; Geoghegan et al., 1983; Auld et al., 1984, 1986), provided that a suitable cryosolvent can be found that does not adversely affect the enzyme. It has been shown previously that LAP is unusually resistant to organic solvents and that experiments can be carried out in 50% v/v methanol without altering the reactions in any fundamental way (Lin & Van Wart, 1982). In this paper, low-temperature stopped-flow fluorescence studies of the pre-steady-state reaction of various [(LAP)Zn_6M_6] with Leu-Gly-NHNH-Dns in 50% v/v methanol are reported. These studies confirm the conclusion of a preliminary report (Van Wart & Lin, 1983) that there are at least two intermediates in the reaction pathway.

Materials and Methods

Materials. The source and purification of the LAP and other materials and the synthesis of Leu-Gly-NHNH-Dns have been described elsewhere (Lin & Van Wart, 1988a,b; Lin et al., 1988). Leu-NHOH was purchased from Sigma Chemical Co. Reagent-grade methanol was purchased from Mallinckrodt.

Measurement of pH and Preparation of Solutions.* The apparent protonic activity in aqueous–organic solutions, pH*, was measured by using an Oreil Model 611 pH meter with a Ross Model 8103 combination glass electrode as described by Fink and Geeves (1979). To prepare a solution with the desired pH* at a subzero temperature in a given buffer and cryosolvent, tables of the temperature dependence of pH* (Douzou, 1977) were used to estimate the change in pH* expected on lowering the temperature from 1 °C to the desired value. The solution was then prepared at 1 °C and the value of pH* adjusted so that, on cooling, the desired pH* would be obtained. For example, to get pH* 9.0 for 50% v/v methanol/10 mM Tris at −5, −10, −20, −30, and −40 °C, the solutions were adjusted to pH* values of 8.80, 8.65, 8.28, 7.90, and 7.48 at 1 °C respectively. All procedures were carried out under metal-free conditions.

Low-Temperature Stopped-Flow Experiments. The stopped-flow fluorescence experiments were carried out as reported in the second of three papers in this issue (Lin et al., 1988). However, to improve performance at subzero temperatures, the original instrument design (Van Wart & Zimmer, 1981) was modified by replacing the Teflon flow module and quartz cell with a Kel-F flow module containing fluid channels flanked by quartz windows (Morris and Van Wart, unpublished results). All solutions were equilibrated in the reactant syringes at the desired temperature for at least 2 h before carrying out mixing experiments. The temperature of the instrument was controlled by pumping precooled methanol from a Neslab Model ULT-80 temperature bath through channels in both sides of the brass block that surrounds the stopped-flow module and reactant syringes. The temperature of the stopped-flow system was measured with an Omega copper/constantan grounded thermocouple and an Omega Model 2176 A-T digital thermometer.

Results

Choice of Cryosolvent and Reaction Conditions. The effects of several cryosolvents on the hydrolysis of Leu-*p*-nitroanilide by LAP have been studied in detail previously (Van Wart & Lin, 1981). The structural and catalytic properties of the enzyme are not adversely affected by cryosolvents containing 50% v/v methanol, ethanol, or dimethyl sulfoxide. Methanol has been chosen as the cosolvent for these studies because of its low viscosity, low freezing point, and high dielectric constant. Since maximal activity in the presence of 50% v/v methanol is achieved in 10 mM Tris/0.1 M KCl at pH* 9, all reactions were carried out under these conditions. The different [(LAP)Zn_6M_6] were prepared as described in the preceding paper (Lin et al., 1988), except that the incubations with the M(II) were carried out directly in 50% v/v methanol/10 mM Tris/0.1 M KCl at the desired pH*. Studies with [(LAP)Zn_6Ni_6] and [(LAP)Zn_6Cu_6] were carried out in the presence of 0.2 mM EDTA, as described earlier (Lin et al., 1988).

Table I: Steady-State Kinetic Parameters for the Hydrolysis of Leu-Gly-NHNH-Dns by [(LAP)Zn_6Mg_6] in the Presence of Various Amounts of Methanol[a]

% v/v methanol	k_{cat} (min^{-1})	K_M (mM)	$(k_{cat}/K_M) \times 10^{-6}$ (min^{-1} M^{-1})
0	1200	0.33	3.6
10	1100	0.32	3.4
20	740	0.26	2.8
30	470	0.24	2.0
40	250	0.24	1.0
50	190	0.27	0.70

[a] All reactions were carried out in 10 mM Tris/0.1 M KCl, pH* 9, containing the indicated percent (v/v) of methanol at 23 °C.

Effect of Methanol on the Steady-State Kinetics of Hydrolysis of Leu-Gly-NHNH-Dns by [(LAP)Zn_6M_6]. The conclusion of an earlier study (Van Wart & Lin, 1982) that methanol has no adverse effects on LAP-catalyzed reactions was based on results obtained by using Leu-*p*-nitroanilide as substrate. Since conclusions regarding the effects of regulatory metal ions on the kinetic parameters for LAP using this substrate differ substantially from those obtained with more specific substrates (Lin et al., 1988), the effect of methanol on the steady-state kinetics of hydrolysis of Leu-Gly-NHNH-Dns has been specifically investigated here as a prerequisite to the low-temperature studies. Stopped-flow fluorescence traces have been obtained for the hydrolysis of Leu-Gly-NHNH-Dns by [(LAP)Zn_6Mg_6] in 10 mM Tris/0.1 M KCl, pH* 9 at 23 °C, in the presence of various percentages of methanol (not shown). Qualitatively, all of these traces are very similar to those obtained in the absence of methanol (Lin et al., 1988), and no new relaxations are observed. The k_{cat} and K_M values for these reactions have been determined by the six methods described previously (Lin et al., 1988). All of the kinetic plots were linear, and the average values of the kinetic parameters derived from these six types of plots are listed in Table I. Since the solubility of Leu-Gly-NHNH-Dns is increased in the presence of methanol ($\sim$10 mM in 50% v/v methanol), the condition $[S_0] > K_M$ has been achieved in these studies.

Increasing percentages of methanol lower k_{cat} approximately linearly and decrease K_M slightly, causing a net decrease in k_{cat}/K_M. The decreases in k_{cat} are almost identical with those observed earlier with Leu-*p*-nitroanilide (Lin & Van Wart, 1982) and are partly attributable to the lowered concentration of water. However, the slight *decrease* in K_M for Leu-Gly-NHNH-Dns caused by methanol contrasts sharply with the sizable *increases* observed earlier. The hydrolysis of Leu-Gly-NHNH-Dns by [(LAP)Zn_6M_6], where M(II) = Mn(II), Ni(II), Cu(II), Zn(II), and empty, in 50% methanol/10 mM Tris/0.1 M KCl, pH* 9 at 23 °C, has also been investigated. The steady-state kinetic parameters for these reactions have been evaluated from the stopped-flow fluorescence traces and are summarized in Table II. These are to be compared with

Table II: Steady-State Kinetic Parameters for the Hydrolysis of Leu-Gly-NHNH-Dns by Various [(LAP)Zn_6M_6] in the Presence of 50% Methanol[a]

enzyme	k_{cat} (min^{-1})	K_M (mM)	$(k_{cat}/K_M) \times 10^{-6}$ (min^{-1} M^{-1})
[(LAP)Zn_6Mg_6]	190	0.27	0.70
[(LAP)Zn_6Mn_6]	160	0.22	0.73
[(LAP)Zn_6Ni_6]	140	0.23	0.61
[(LAP)Zn_6Cu_6]	120	0.21	0.57
[(LAP)Zn_6Zn_6]	90	0.18	0.50
[(LAP)Zn_6—]	80	0.11	0.73

[a] All reactions were carried out in 50% v/v methanol/10 mM Tris/0.1 M KCl, pH* 9 at 23 °C.

the corresponding results obtained in the absence of methanol, which are listed in Table II of Lin et al. (1988). The values of k_{cat} are between 2- and 12-fold lower in the presence of 50% v/v methanol for these [(LAP)Zn_6M_6]. In contrast, the values of K_M are almost unaffected. The net result is a lowering of the k_{cat}/K_M values for all of the metallohybrids. These results are consistent with the view that methanol has no fundamental effect on the hydrolysis of Leu-Gly-NHNH-Dns by [(LAP)Zn_6M_6] under these conditions. In fact, methanol causes a much smaller reduction in activity toward Leu-Gly-NHNH-Dns than Leu-*p*-nitroanilide for all of the [(LAP)-Zn_6M_6].

Pre-Steady-State Kinetics of Reaction of Leu-Gly-NHNH-Dns by [(LAP)Zn_6M_6] at Subzero Temperatures. The pre-steady-state reaction of Leu-Gly-NHNH-Dns with [(LAP)Zn_6M_6], where M(II) = empty, Ni(II), Cu(II), Mg(II), and Zn(II), has been studied in 50% v/v methanol/10 mM Tris/0.1 M KCl, pH* 9, at subzero temperatures where the conversion of S to P is negligible. Since energy-transfer experiments cannot be used to detect the ES_i for this enzyme–substrate system, direct dansyl excitation has been employed.

Lobb and Auld (1984) have shown that the fluorescence changes, F_t, observed during the pre-steady-state reaction of an E with a fluorescent S due to direct excitation of reactants is given by

$$F_t = C_0[S_0] + C\sum(Q_i a_i - Q_S a_S)[ES_i] \quad (1)$$

where C_0 and C are constants, $[S_0]$ is the total substrate concentration, and $Q_i a_i$ and $Q_S a_S$ are the products of the quantum yield and absorbance of the dansyl group of ES_i and S, respectively, at the excitation wavelength. If the time between the acquisition of consecutive stopped-flow traces is short and no product (P) is formed, the $C_0[S_0]$ term will not contribute to the traces and can be effectively neglected. This relationship predicts that if any intermediate ES_i has a value of $Q_i a_i$ that is different from that of S, changes in its concentration will lead to nonzero values of F_t. Thus, the pre-steady-state changes in the concentration of ES_i can potentially be detected from stopped-flow fluorescence studies under nonturnover conditions. It has also been shown by Lobb and Auld (1984) that F_t can be expressed as a series of exponential terms which, neglecting the $C_0[S_0]$ term for the reasons described above, has the form

$$F_t = \sum_{j=1}^{n} C_j e^{-k_j t} \quad (2)$$

where

$$C_j = \sum_{i=1}^{n} C_i A_{ij} \quad (3)$$

Thus, the F_t for an elementary step well separated in time from others will give rise to relaxations that exhibit exponential behavior and be described by a first-order rate constant, k_{obsd}.

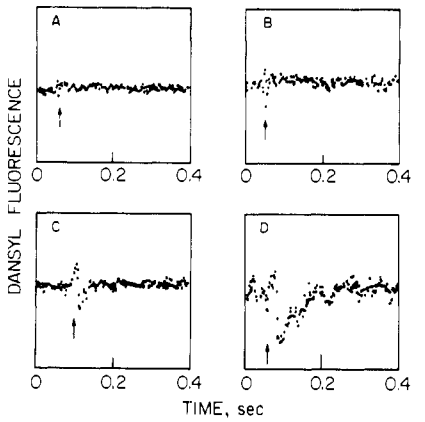

FIGURE 1: Stopped-flow fluorescence traces (λ_{ex} = 320 nm) for the pre-steady-state reaction of 0.2 μM [(LAP)Zn_6Ni_6] with 0.1 mM Leu-Gly-NHNH-Dns in 50% v/v methanol/10 mM Tris/0.1 M KCl, pH* 9, containing 0.1 mM Ni(II) and 0.2 mM EDTA at (A) 0, (B) −10, (C) −26, and (D) −35 °C. The arrow indicates the onset of mixing.

Stopped-flow fluorescence traces for the reaction of [(LAP)Zn_6Ni_6] with Leu-Gly-NHNH-Dns in 50% v/v methanol/10 mM Tris/0.1 M KCl, pH* 9, containing 0.1 mM $NiCl_2$ and 0.2 mM EDTA between 0 and −35 °C are shown in Figure 1. These traces were recorded with a high sensitivity, and the slightest turbulence or vibration caused by mixing can affect the fluorescence signal. Thus, as a stringent control against artifacts, all traces were recorded by using a pretrigger (the onset of mixing is indicated by the vertical arrows in Figure 1) so that all changes in fluorescence that occurred both during and after mixing could be seen. At temperatures above −26 °C, no pre-steady-state relaxations are detected with an excitation wavelength at 320 nm (Figure 1A,B) (or 280 nm, data not shown). There is an indication of a relaxation at −26 °C (Figure 1C), but its amplitude is small. At −35 °C, however, this new relaxation with a negative value of F_0 is clearly seen (Figure 1D). This relaxation is reproducible and is absent in control experiments in which E and buffer, or S and buffer, were used as reactants. In addition, it is absent in experiments with E that had been preincubated with the competitive inhibitor Leu-NHOH (200 μM; K_I = 33 μM in this cryosolvent at 23 °C), indicating that the relaxation involves a species of S that is bound at the active site of E. Stopped-flow experiments with E and Dns-NH-$(CH_2)_2NH_2$ also do not show this relaxation, providing further support that it does not arise from nonspecific binding.

At −35 °C, there is essentially no P formation during the acquisition time of a stopped-flow trace. It was established in an earlier study (Lin & Van Wart, 1982) from an Arrhenius plot that lowering the temperature from 25 to −30 °C reduces k_{cat} approximately 27-fold. Since k_{cat} for [(LAP)Zn_6Ni_6] at 23 °C is 140 min^{-1} (Table II), it is calculated to be no greater than 5.1 min^{-1} at −35 °C. Thus, one round of catalysis takes approximately 12 s when E is fully saturated with S. Hence, the conditions used to obtain the trace shown in Figure 1D can effectively be considered nonturnover conditions. Importantly, the Arrhenius plot obtained earlier for k_{cat} over the 25 to −30 °C range is linear, indicating that the reduction in rate is attributable solely to the lowered temperature and not to changes in enzyme conformation or rate-determining step.

Under nonturnover conditions, the values of F_t are described by eq 1 and are attributable to changes in the concentration of at least one ES_i. Equation 1 predicts that F_0, the maximal fluorescence for this relaxation, is proportional to $[ES_i]$. Since

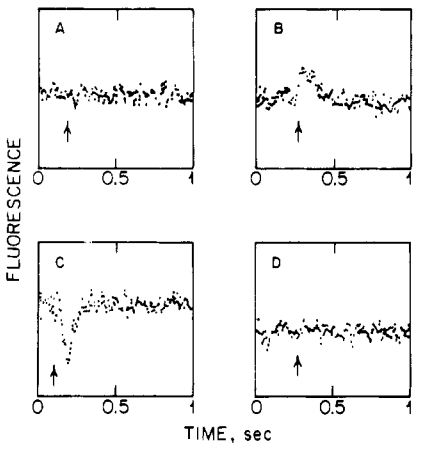

FIGURE 2: Stopped-flow fluorescence traces for the reaction of 0.2 μM [(LAP)Zn_6Ni_6] with 0.1 mM Leu-Gly-NHNH-Dns in 50% v/v methanol/10 mM Tris/0.1 M KCl, pH* 9, containing 0.1 mM Ni(II) and 0.2 mM EDTA at −35 °C, showing the changes in dansyl emission on excitation at (A) 260, (B) 280, and (C) 330 nm and (D) the change in Trp emission on excitation at 280 nm. The arrow indicates the onset of mixing.

these experiments are carried out under conditions where $[E_0] \ll [S_0]$, F_0 should also be proportional to $[E_0]$. A plot of F_0 as a function of $[E_0]$ over a range of concentrations from 0 to 0.20 μM for the reaction of 0.1 mM Leu-Gly-NHNH-Dns with [(LAP)Zn_6Ni_6] at −35 °C has been constructed (not shown). The plot shows that F_0 is directly proportional to $[E_0]$ and intersects the origin, confirming that this relaxation is due to at least one enzyme-bound form of S.

Equation 1 also predicts that the sign of F_t for a pre-steady-state relaxation can change when recorded at different excitation wavelengths. For example, for a relaxation involving the equilibration of free S with ES_i, F_t would be positive at excitation wavelengths at which $Q_i a_i > Q_S a_S$ and negative at wavelengths where $Q_i a_i < Q_S a_S$. This situation could well occur, for example, if the spectrum of the fluorescent S were shifted in ES_i relative to free S. A similar change in sign of F_t with excitation wavelength can occur for pre-steady-state relaxations due to the interconversion of two ES_i if the relative values of $Q_i a_i - Q_S a_S$ for two different ES_i are reversed.

In order to investigate this possibility, the maximum fluorescence change, F_0, for the pre-steady-state relaxation observed on reaction of [(LAP)Zn_6Ni_6] with Leu-Gly-NHNH-Dns at −35 °C has been measured at different excitation wavelengths. Stopped-flow traces obtained with excitation at 260, 280, and 330 nm are shown in panels A, B, and C, respectively, of Figure 2. It can be seen that F_0 is zero, positive, and negative at these three excitation wavelengths, respectively. A stopped-flow fluorescence excitation profile consisting of F_0/F_{bkg} plotted as a function of excitation wavelength every 10 nm from 260 to 360 nm is shown in Figure 3. This profile exhibits a negative peak at 330 nm and a positive peak at 280 nm and shows that the sign of F_0 is reversed when the excitation wavelength is lower than 285 nm. The shape of this profile is that expected for a difference spectrum, indicating that the new relaxation corresponds to the interconversion of two forms of S in which the emission and/or absorption spectra of the dansyl group are shifted. Evidence against there being any contribution to this fluorescence change due to E-to-S energy transfer is the observation that no corresponding relaxation due to changes in Trp emission has been detected (Figure 2D).

In principle, this pre-steady-state relaxation observed for the reaction of Leu-Gly-NHNH-Dns with [(LAP)Zn_6Ni_6] can be attributed to any elementary step involving the equilibration

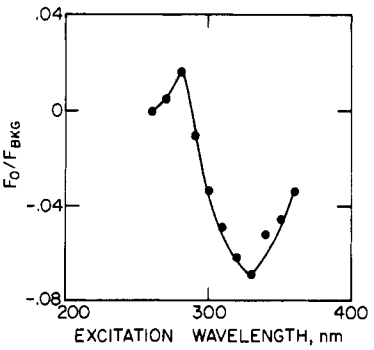

FIGURE 3: Stopped-flow fluorescence excitation profile for the reaction of 0.2 μM [(LAP)Zn_6Ni_6] with 0.1 mM Leu-Gly-NHNH-Dns in 50% v/v methanol/10 mM Tris/0.1 M KCl, pH* 9, containing 0.1 mM Ni(II) and 0.2 mM EDTA at −35 °C.

of two forms of S, at least one of which is enzyme-bound. The relationship between k_{obsd} for this relaxation and the concentration of reactants depends on the type of elementary step to which the relaxation corresponds. The three most likely possibilities have been considered by Lobb and Auld (1984) and are recounted below. The first is the case where the relaxation observed corresponds only to the equilibration of E and S to form ES where subsequent decay of ES is very slow:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{\text{slow}}{\rightleftharpoons} \ldots \quad (4)$$

When $[S_0] \gg [E_0]$, the observed first-order rate constant for this reaction is given by

$$k_{obsd} = k_{-1} + k_1[S_0] \quad (5)$$

The second example is that of a rapid preequilibrium between S and ES_1 followed by an isomerization of ES_1 to ES_2

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES_1 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ES_2 \ldots \quad (6)$$
(fast)

for which

$$k_{obsd} = k_{-2} + k_2\frac{[S_0]}{K_s + [S_0]} \quad (7)$$

where $K_s = k_{-1}/k_1$. The third example involves a slow conformational change in E prior to the binding of S

$$E' \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E + S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ES \ldots \quad (8)$$
(fast)

for which

$$k_{obsd} = k_1 + k_{-1}\frac{K_s}{K_s + [S_0]} \quad (9)$$

where $K_s = k_{-2}/k_2$. In each of these three mechanisms, k_{obsd} exhibits a different dependence on $[S_0]$, which can be used to distinguish between them experimentally. Equation 5 predicts that k_{obsd} will increase linearly with $[S_0]$. Equation 7 predicts that k_{obsd} will increase with $[S_0]$ but saturate when $[S_0] \gg K_s$. Equation 9 predicts that k_{obsd} will decrease as $[S_0]$ increases.

In order to establish the origin of the pre-steady-state relaxation shown in Figure 1, low-temperature stopped-flow fluorescence traces for the reaction of 0.2 μM [(LAP)Zn_6Ni_6] with Leu-Gly-NHNH-Dns in 50% v/v methanol/10 mM Tris/0.1 M KCl, pH* 9, containing 0.1 mM $NiCl_2$ and 0.2 mM EDTA at −35 °C have been obtained at various concentrations of S (Figure 4). Each of the traces is fit well by the single exponential shown as the solid curve. The values of k_{obsd} for these traces are plotted as a function of $[S_0]$ in

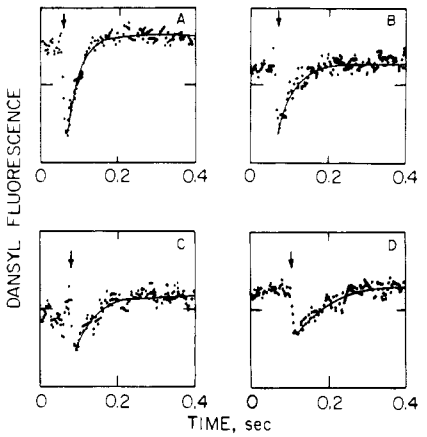

FIGURE 4: Low-temperature stopped-flow fluorescence traces (data points) (λ_{ex} = 330 nm) and their best-fit single exponential curves (solid lines) for the reactions of 0.2 μM [(LAP)Zn_6Ni_6] with various concentrations of Leu-Gly-NHNH-Dns in 50% v/v methanol/10 mM Tris/0.1 M KCl, pH* 9, containing 0.1 mM Ni(II) and 0.2 mM EDTA at −35 °C. The arrow indicates the onset of mixing. The substrate concentration and the observed rate constant for each trace are as follows: (A) $[S_0]$ = 0.25 mM, k_{obsd} = 30 s^{-1}; (B) $[S_0]$ = 0.15 mM, k_{obsd} = 23 s^{-1}; (C) $[S_0]$ = 0.10 mM, k_{obsd} = 18.5 s^{-1}; (D) $[S_0]$ = 0.05 mM, k_{obsd} = 12.5 s^{-1}.

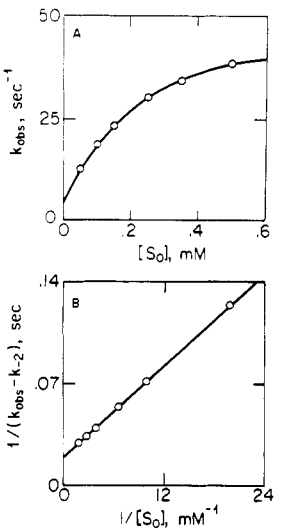

FIGURE 5: Plots of (A) k_{obsd} versus $[S_0]$ and (B) $1/(k_{obsd} - k_{-2})$ versus $1/[S_0]$ for the reaction of 0.2 μM [(LAP)Zn_6Ni_6] with various concentrations of Leu-Gly-NHNH-Dns in 50% v/v methanol/10 mM Tris/0.1 M KCl, pH* 9, containing 0.1 mM Ni(II) and 0.2 mM EDTA at −35 °C. The pre-steady-state kinetic parameters estimated from these plots are k_2 = 54 s^{-1}, k_{-2} = 4.4 s^{-1}, and K_s = 0.28 mM.

Figure 5A. It can clearly be seen that k_{obsd} increases as a function of $[S_0]$ and approaches saturation at high $[S_0]$. Thus, this new relaxation is due to the interconversion of ES_1 and ES_2, where ES_1 is an unstable intermediate that is only observed at low temperature.

Rearrangement of eq 7 leads to

$$\frac{1}{k_{obsd} - k_{-2}} = \frac{1}{k_2} + \frac{K_s}{k_2}\frac{1}{[S_0]} \qquad (10)$$

which is the basis for a linear double-reciprocal plot for determining the pre-steady-state kinetic parameters. A plot of $1/(k_{obsd} - k_{-2})$ versus $1/[S_0]$ should be linear with a slope of K_s/k_2 and y intercept of $1/k_2$. To construct such a plot, k_{-2} is estimated by extrapolating the curve of k_{obsd} versus $[S_0]$ shown in Figure 5A to an $[S_0]$ value of zero. The plot of $1/(k_{obsd} - k_{-2})$ versus $1/[S_0]$ obtained in this manner is shown in Figure 5B. From the slope and intercept, the kinetic parameters are k_2 = 54 s^{-1}, k_{-2} = 4.4 s^{-1}, and K_s = 0.28 mM.

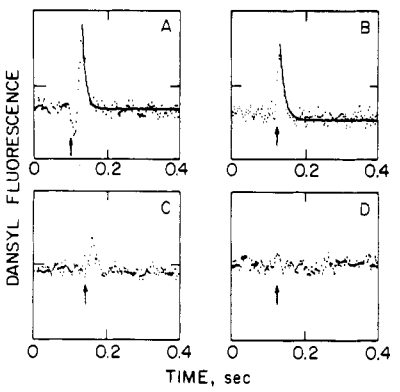

FIGURE 6: Stopped-flow fluorescence traces (λ_{ex} = 320 nm) for the reaction of 50 μM Leu-Gly-NHNH-Dns with 0.2 μM (A) [(LAP)Zn_6Cu_6], (B) [(LAP)Zn_6Zn_6], (C) [(LAP)Zn_6Mg_6], and (D) [(LAP)Zn_6—] in 50% v/v methanol/10 mM Tris/0.1 M KCl, pH* 9 at −35 °C. The arrow indicates the onset of mixing. The best-fit single exponential curves with k_{obsd} = 100 and 80 s^{-1} are shown for the traces in (A) and (B), respectively.

Similar low-temperature stopped-flow experiments for the reaction of Leu-Gly-NHNH-Dns with other [(LAP)Zn_6M_6], where M = empty, Cu(II), Zn(II), and Mg(II), have also been carried out in 50% v/v methanol/10 mM Tris/0.1 M KCl, pH* 9, between 0 and −35 °C. No relaxations are observed at temperatures above −26 °C for any of these reactions. However, at −35 °C, a new relaxation is visible for [(LAP)Zn_6Cu_6] and [(LAP)Zn_6Zn_6] that is just longer than the mixing time (approximately 20 ms) of the instrument (Figure 6A,B). The values of k_{obsd} for these relaxations are 100 and 80 s^{-1} for [(LAP)Zn_6Cu_6] and [(LAP)Zn_6Zn_6], respectively. There is a suggestion of a similar relaxation for [(LAP)Zn_6Mg_6], but with considerably smaller amplitude (Figure 6C). While these relaxations are reproducible and are absent in control experiments without E, they are just barely resolvable and a quantitative study of them is not possible. Interestingly, the direction of the fluorescence change for both [(LAP)Zn_6Cu_6] and [(LAP)Zn_6Zn_6] is opposite to that of [(LAP)Zn_6Ni_6] with excitation at 320 nm. No new relaxation has been detected for the reaction of Leu-Gly-NHNH-Dns with [(LAP)Zn_6—] even at −35 °C (Figure 6D).

Discussion

In this paper, the resolving power of stopped-flow cryoenzymology has been exploited in order to study the pre-steady-state reaction of various [(LAP)Zn_6M_6] with Leu-Gly-NHNH-Dns. These studies are dependent on having a cryosolvent that does not adversely alter the course of the enzymatic reaction under study. It has been shown previously that methanol was a suitable cosolvent that did not markedly alter the steady-state kinetics of hydrolysis of Leu-*p*-nitroanilide by this enzyme. However, since the results of the preceding paper (Lin et al., 1988) suggested that conclusions based on the kinetics of this relatively poor substrate could be misleading, the effect of methanol on the hydrolysis of Leu-Gly-NHNH-Dns at ambient temperature was specifically examined before attempting experiments at lower temperatures.

Interestingly, while it was found earlier that methanol lowered k_{cat} and raised K_M for the hydrolysis of Leu-*p*-nitroanilide, it lowers k_{cat} but has almost no effect on K_M for Leu-Gly-NHNH-Dns. Since K_M is approximately equal to K_s for these reactions, methanol apparently weakens the binding of Leu-*p*-nitroanilide due to hydrophobic partitioning between the enzyme and cryosolvent, an effect that is attrib-

utable to the nitroanilide group. The more physiologically relevant substrate Leu-Gly-NHNH-Dns has true enzyme–substrate interactions in subsites P_1 and P_1' [nomenclature of Schechter and Berger (1967)], which are less dependent on hydrophobic interactions than for Leu-*p*-nitroanilide and are almost unaffected by methanol. Methanol lowers k_{cat} for Leu-Gly-NHNH-Dns and Leu-*p*-nitroanilide almost equally. Over half of the sixfold lowering for [(LAP)Zn_6Mg_6] by 50% v/v methanol, however, can be attributed to the decreased concentration of water, which is a coreactant. Thus, while the earlier conclusion (Lin & Van Wart, 1982) that methanol does not affect the catalytic reaction between LAP and Leu-*p*-nitroanilide in any fundamental way is correct, the trends observed here with the more specific substrate Leu-Gly-NHNH-Dns are somewhat different and even more convincingly support this conclusion.

The low-temperature stopped-flow fluorescence studies with several [(LAP)Zn_6M_6] have uncovered a new relaxation at −35 °C. For [(LAP)Zn_6Ni_6], the relaxation was slow enough that its characteristics could be studied in detail. In particular, it has clearly been shown to arise from the interconversion of two ES_i. Presumably, the same is true for [(LAP)Zn_6Cu_6] and [(LAP)Zn_6Zn_6], although this cannot be stated with certainty. Thus the mechanism of interaction of LAP with Leu-Gly-NHNH-Dns entails at least two reaction intermediates as shown:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES_1 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ES_2 \xrightarrow{k_3} E + P \qquad (11)$$

This mechanism can account for all of the observations in this study. In all of the stopped-flow experiments, the combination of E and S to form ES_1 occurs during mixing, and no relaxation due to this step is ever resolved. Between −26 and 0 °C, there is neither any turnover on the time scale of the stopped-flow experiment nor any resolution of the ES_1 to ES_2 interconversion for any of the [(LAP)Zn_6M_6]. Thus, the stopped-flow traces are flat. At −35 °C, however, a relaxation corresponding to the interconversion of ES_1 and ES_2 can be detected for [(LAP)Zn_6Ni_6], [(LAP)Zn_6Cu_6], and [(LAP)Zn_6Zn_6].

A pictorial representation of the stopped-flow fluorescence traces for the reaction of Leu-Gly-NHNH-Dns with [(LAP)Zn_6Ni_6] obtained with 320- and 280-nm excitation is given in Figure 7. Traces A and C are curves obtained with 320- and 280-nm excitation, respectively, for a hypothetical reaction in which the time between experiments is long enough to allow all of the S, ES_1, and ES_2 to decompose to E + P. Traces B and D are curves obtained with excitation at 320 and 280 nm, respectively, under nonturnover conditions in which the starting value of the fluorescence is that of the S and ES_2 left over in the observation cell from the previous experiment. These traces mimic the situation for the low-temperature stopped-flow experiments carried out here. Note that when the excitation wavelength is 320 nm (Figure 7A,B), ES_2 has a higher fluorescence relative to S than ES_1 ($Q_2a_2 - Q_Sa_S$ is larger than $Q_1a_1 - Q_Sa_S$ in eq 1) and the value of F_0 measured in the low-temperature stopped-flow experiments is negative. In contrast, when the excitation wavelength is 280 nm (Figure 7C,D), ES_1 has the higher fluorescence and the values of F_0 are positive. Thus, the fluorescence changes observed under nonturnover conditions for [(LAP)Zn_6Ni_6] reflect the interconversion of ES_1 and ES_2, and their magnitude and sign are determined by the spectral properties of S in these two species. Assuming that the relaxations observed for [(LAP)Zn_6Cu_6] and [(LAP)Zn_6Zn_6] also rise from this same interconversion, the opposite sign of F_0 observed for these processes compared

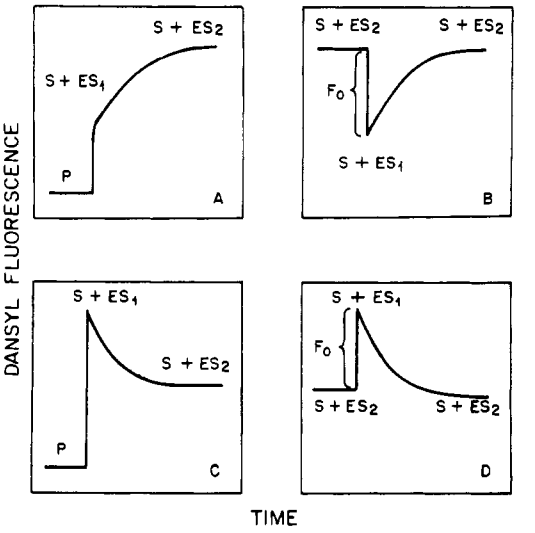

FIGURE 7: Pictorial representations of stopped-flow fluorescence traces showing how changes in the concentrations of fluorescent species lead to different patterns for the hydrolysis of Leu-Gly-NHNH-Dns by [(LAP)Zn_6Ni_6] at −35 °C. Traces A and B correspond to λ_{ex} = 320 nm where the fluorescence of ES_1 is lower than that of ES_2, while traces C and D correspond to λ_{ex} = 280 nm where the fluorescence of ES_1 is greater than that of ES_2. Traces A and C are the hypothetical traces that would be obtained if time between experiments is long enough for ES_2 to completely decay to E + P. Traces B and D are those actually observed under nonturnover conditions in which the starting value of the fluorescence is that of undecayed ES_2.

to [(LAP)Zn_6Ni_6] with 320-nm excitation indicates that the spectral properties of S in ES_1 and ES_2 differ for these metallohybrids.

The conclusions of this study are in good agreement with preliminary results reported earlier (Van Wart & Lin, 1983). The present investigation differs slightly, however, in that extensive background studies (Lin & Van Wart, 1988a,b; Lin et al., 1988) have indicated more optimal conditions for these experiments, including the use of EDTA in the reactions with [(LAP)Zn_6Ni_6] and [(LAP)Zn_6Cu_6] and excitation near 320 nm for greater sensitivity. The studies with [(LAP)Zn_6Mg_6] carried out here at −35 °C (Figure 5C) have been unable to clearly resolve the relaxation observed earlier at −40 °C. In the previous study, it was thought that the differences in fluorescence of ES_1 and ES_2 were due to differences in the extent of energy transfer in each. However, it is now clear that these differences are due to the different environments of the dansyl group of S in these two intermediates, as reflected by their different values of Q_ia_i. Further studies will be needed to provide specific information about the structures of ES_1 and ES_2. However, this study demonstrates the unique potential of stopped-flow cryoenzymology for the detection and study of unstable catalytic intermediates.

Registry No. LAP, 9001-61-0; Leu-Gly-NHNH-Dns, 89315-19-5; methanol, 67-56-1.

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Interaction of the Cysteine Proteinase Inhibitor Chicken Cystatin with Papain†

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Received December 28, 1987; Revised Manuscript Received March 10, 1988

ABSTRACT: The two forms of chicken cystatin, with different isoelectric points, that have been described previously were indistinguishable in analyses of amino- and carboxy-terminal residues, amino acid composition, and peptide maps. The two forms thus are highly similar and most likely differ only in an amide group or in a small charged substituent. The binding of either cystatin form to highly purified, active papain was accompanied by the same pronounced changes in near-ultraviolet circular dichroism, ultraviolet absorption, and fluorescence emission. These changes were compatible with perturbations of the environment of aromatic residues in one or both proteins of the complex, arising from local interactions or from a conformational change. Modification of the single tryptophan residue of cystatin, at position 104, with *N*-bromosuccinimide resulted in considerably smaller spectroscopic changes on binding of the inhibitor to papain, indicating that the environment of this residue is affected by the binding. Analogous modification of Trp-69 and Trp-177 of papain markedly affected the fluorescence changes observed on binding of cystatin to the enzyme, similarly suggesting that these two residues of papain are involved in the interaction. The fluorescence increase of papain at alkaline pH, arising from Trp-177 and due to deprotonization of the adjacent His-159, was abolished on binding of cystatin to the enzyme, further supporting the proposal that this region of papain participates in the interaction with the inhibitor. A stoichiometry of binding of either cystatin form to papain of 1:1 and a lower limit for the binding constant of 10^9 M^{-1} were determined by titrations monitored by either the ultraviolet absorption or fluorescence changes induced by the interaction.

Protein inhibitors of cysteine proteinases are widely distributed in mammalian tissues and are present also in plasma. Their function presumably is to protect the organism against uncontrolled action of endogenous or exogenous cysteine proteinases (Barrett et al., 1986). Those inhibitors of this group that are of tissue origin, cystatins, are small proteins with M_r 12000-14000. To date, four such cystatins, A, B, C, and S, have been demonstrated in human tissues and secretions, and analogues to some of these have been shown to occur also in other mammals (Barrett et al., 1986). The cysteine proteinase inhibitors of plasma are identical with the kininogens (Okhubo et al., 1984; Müller-Esterl et al., 1985; Sueyoshi et al., 1985), which exist in two partially identical forms, H-kininogen and L-kininogen with M_r ~100000 and 60000, respectively (Kato et al., 1981; Lottspeich et al., 1985; Kellerman et al., 1986). The kininogens also have other biological activities. Both forms are thus precursor molecules

†Supported by grants from the Swedish Medical Research Council (Projects 4212 and 3532), the Swedish Council for Forestry and Agricultural Research (Project 600/83), the Swedish Cancer Society (Project 1806), and Konung Gustaf V:s 80-årsfond.
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0006-2960/88/0427-5074$01.50/0