Steady-State Kinetics of Hydrolysis of Dansyl-Peptide Substrates by Leucine Aminopeptidase†

Wann-Yin Lin, Spencer H. Lin, and Harold E. Van Wart*

Department of Chemistry and Institute of Molecular Biophysics, Florida State University, Tallahassee, Florida 32306

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ABSTRACT: Stopped-flow fluorescence experiments have been carried out at 23 °C to study the hydrolysis of Leu-Gly-NHNH-Dns [Dns = 5-(dimethylamino)naphthalene-1-sulfonyl] and Leu-Gly-NH$(CH_2)_2$NH-Dns by porcine kidney cytosol leucine aminopeptidase (LAP). Experiments have been performed with LAP species containing Mg(II), Mn(II), Ni(II), Cu(II), Zn(II), and no metal ion at the regulatory metal binding site. The fluorescence changes observed on hydrolysis of these dansyl substrates by LAP arise from changes in the concentration of substrate. Several kinetic relationships have been developed that allow the steady-state kinetic parameters for these reactions to be determined from the stopped-flow fluorescence traces. When any of the five metal ions are bound at the regulatory site, k_{cat} and K_M are both raised to approximately the same extent with the result that the maximum increase observed for k_{cat}/K_M is only approximately twofold. The effects of these metal ions on k_{cat}, K_M, and k_{cat}/K_M observed for these substrates differ markedly from those for less physiologically relevant substrates, such as Leu-*p*-nitroanilide, that do not have amino acids on both sides of the scissile bond. This suggests that earlier conclusions regarding the effect of the regulatory metal ion on the activity of LAP may have been misleading and casts doubt as to whether the term "regulatory site" has validity in the context of LAP-catalyzed reactions under physiological conditions.

Porcine kidney cytosol leucine aminopeptidase (LAP)[1] (EC 3.4.11.1) was one of the first N-terminal metallopeptidases to be studied in any detail (Delange & Smith, 1971; Himmelhoch, 1970). It is similar in many ways to the aminopeptidase from bovine lens (Taylor et al., 1984a,b; Oettgen & Taylor, 1985) that has been studied more extensively. In particular, both aminopeptidases are hexameric and possess two metal binding sites per subunit (Carpenter & Vahl, 1973; Thompson & Carpenter, 1976a,b; Van Wart & Lin, 1981; Allen et al., 1983). The activity of both aminopeptidases is influenced by the identity of the metal ion that resides at each site (Carpenter & Vahl, 1973; Thompson & Carpenter, 1976a; Van Wart & Lin, 1981; Allen et al., 1983), and it is of interest to differentiate the role of the metal ion at these two binding sites in catalysis.

After chromatographic purification, porcine kidney and bovine lens LAP contain one and two Zn(II) atoms per subunit, respectively (Carpenter & Vahl, 1973; Van Wart & Lin, 1981). Porcine kidney LAP also binds a second Zn(II) per subunit in the presence of excess Zn(II), but the association constant is too small to allow isolation of the enzyme containing both Zn(II) atoms. Removal of the single Zn(II) atom from each subunit of porcine kidney LAP and of both Zn(II) atoms from bovine lens LAP abolishes activity for both enzymes. This indicates that at least one Zn(II) atom is catalytically essential for each enzyme and implies that it is located at the active site. This will be referred to here as the catalytic metal binding site, although this has alternately been referred to as the specificity site (Carpenter & Vahl, 1973) or structural site (Thompson & Carpenter, 1976a) for bovine lens LAP. Porcine kidney LAP with Zn(II) at the six catalytic sites and the second site vacant is designated [(LAP)Zn_6—], while the native form of the bovine lens enzyme is designated [(LAP)Zn_6Zn_6].

Incubation of both aminopeptidases with either Mg(II) or Mn(II) results in the incorporation of one of these ions per subunit with an accompanying increase in activity (Carpenter & Vahl, 1973; Van Wart & Lin, 1981). For porcine kidney LAP, these ions bind at the second site on each subunit to form the metallohybrids [(LAP)Zn_6Mg_6] and [(LAP)Zn_6Mn_6], respectively. These same species are formed for bovine lens LAP but require the exchange of Mg(II) or Mn(II) for the Zn(II) in the second metal binding site. This site has been called the activation site (Carpenter & Vahl, 1973) because of the stimulation in activity brought about by Mg(II) and Mn(II) for bovine lens LAP. However, since it has been shown for porcine kidney LAP that ions such as Cu(II) and Ni(II) bind to this site to lower activity (Van Wart & Lin, 1981), the more general term regulatory site has been proposed. Since there is no precedent for Mg(II) serving as a catalytic metal by direct interaction with peptide substrates, it probably influences the activity of the enzyme by altering its conformation. Recent studies by Taylor et al. (1982) on a complex between bovine lens [(LAP)Zn_6M_6] and a competitive inhibitor indicate that Mn(II) present at the regulatory site is in close proximity to the inhibitor at the active site. This implies that the catalytic and regulatory sites on each subunit are in close proximity.

All of the conclusions detailed above pertaining to the influence of the metal ions at the two binding sites of these LAP have been obtained from steady-state kinetic studies that employed either Leu-NH_2 or Leu-anilides as substrates (Car-

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*Author to whom correspondence should be addressed at the Institute of Molecular Biophysics.

[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; E, enzyme; S, substrate; P, product; ES, enzyme–substrate intermediate.

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penter & Vahl, 1973; Thompson & Carpenter, 1976a,b; Van Wart & Lin, 1981; Allen et al., 1983). In fact, the differentiation and functional description of these two sites are based almost totally on these kinetic studies. Kinetic data for the hydrolysis of more physiologically relevant substrates, particularly those in which subsite P'_1 [nomenclature of Schechter and Berger (1967)] is occupied by an amino acid, have been scarce (Delange & Smith, 1971). This is due in large part to the difficulty of carrying out accurate kinetic measurements with such substrates, since the rates of their hydrolysis are not amenable to measurement by convenient spectrometric assays. In this paper, the stopped-flow fluorescence technique has been used to study the steady-state kinetics of hydrolysis of Leu-Gly-NHNH-Dns by several [(LAP)Zn_6M_6] in order to better define the influence of the regulatory metal ion on the kinetic parameters k_{cat} and K_M. The results differ from those obtained when Leu-*p*-nitroanilide is used as substrate and suggest that kinetic information not obtained with physiologically relevant substrates can be misleading. In the following paper in this issue (Lin et al., 1988), the pre-steady-state kinetics of these reactions are also examined.

Materials and Methods

Materials. LAP was obtained from Sigma Chemical Co. (type III-CP) or Accurate Chemical and Science Co. and purified by chromatography over Leu-Gly-AH-Sepharose to give [(LAP)Zn_6—] as described previously (Van Wart & Lin, 1981). Leu-*p*-nitroanilide was also purchased from Sigma Chemical Co. The synthesis of Leu-Gly-NHNH-Dns and Leu-Gly-NH$(CH_2)_2$NH-Dns have been described earlier (Lin & Van Wart, 1988b).

Preparation of [*(LAP)Zn_6M_6*]. The metallohybrids [(LAP)Zn_6M_6] were prepared by incubation of [(LAP)Zn_6—] with the chloride or sulfate salts of the various M(II) in 10 mM Tris at 37 °C for 4 h in accordance with earlier findings (Van Wart & Lin, 1981). The M(II) concentrations in the incubation solutions were 0.1 mM for Zn(II), Ni(II), and Cu(II) but 5 mM for Mg(II) and Mn(II). The pH of the incubation solution was 8.0 for all ions except for Mg(II), which was incubated at pH 8.8. Kinetic experiments with [(LAP)Zn_6Cu_6] and [(LAP)Zn_6Ni_6] were carried out in the presence of 0.2 mM EDTA, as described in the text. All manipulations were carried out under metal-free conditions using Johnson-Matthey spectrographically pure metal salts as described in the preceding paper in this issue (Lin & Van Wart, 1988a).

Kinetic Measurements. Assays for the hydrolysis of Leu-*p*-nitroanilide were carried out spectrophotometrically by continuously monitoring the appearance of *p*-nitroaniline at 405 nm with a Varian Model 219 spectrophotometer. Assays were carried out at a substrate concentration of 1 mM in 10 mM Tris and 0.1 M KCl, pH 8.0 [K_M = 1 mM (Van Wart & Lin, 1981)]. Initial velocities were calculated from the slope of the absorbance change during the first 10% of hydrolysis. The concentrations of Leu-*p*-nitroanilide and the product, *p*-nitroaniline, were calculated by using $\epsilon_{320} = 1.4 \times 10^4$ M^{-1} cm^{-1} and $\epsilon_{405} = 9.9 \times 10^3$ M^{-1} cm^{-1}, respectively. The stopped-flow fluorescence experiments were carried out as described earlier (Lin & Van Wart, 1988a) by using an excitation wavelength of 320 nm and an Oriel 420-nm long-pass filter to select the dansyl fluorescence. The concentrations of LAP hexamers and all dansyl substrates were also measured as before (Lin & Van Wart, 1988a).

Theory

In the preceding paper (Lin & Van Wart, 1988a), it was established that the stopped-flow fluorescence changes observed

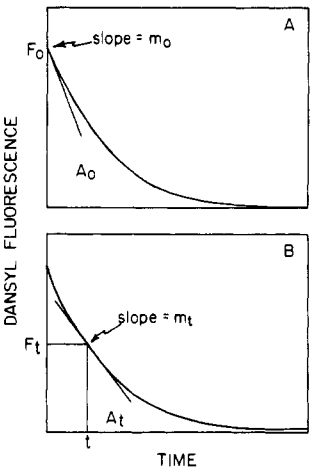

FIGURE 1: Schematic illustration of the variation of dansyl fluorescence with time in a stopped-flow fluorescence experiment, illustrating the definitions of (A) F_0, A_0, and m_0 and (B) F_t, A_t, and m_t.

for the hydrolysis of dansyl substrates by LAP are given by a relationship derived by Lobb and Auld (1984)

$$F_t = C'[S]_t \tag{1}$$

where $C' = C(Q_S a_S - Q_P a_P)$, Q_S and a_S are the quantum yield and molar absorptivity, respectively, of the dansyl group of the substrate (S) at the excitation wavelength, and Q_P and a_P are the same for the product (P). Thus, C' is a constant in any given experiment, and as long as the fluorescence of S is proportional to its concentration (i.e., no self-absorption, etc.), F_t simply reflects the instantaneous concentration of free S.

In order to use the stopped-flow traces to abstract kinetic information, a relationship between the stopped-flow fluorescence parameters and the steady-state kinetic parameters k_{cat} and K_M is desired. This is obtained by starting with the form of the Michaelis–Menten equation that gives the instantaneous rate in a stopped-flow experiment, v_t, as

$$v_t = -\frac{d[S]_t}{dt} = \frac{k_{cat}[E_0][S]_t}{K_M + [S]_t} \tag{2}$$

where $[E_0]$ is the total enzyme concentration and $[S]_t$ is the free substrate concentration at time t. Since consideration is restricted here to circumstances under which $[E_0] \ll [S_0]$, $[S]_t = [S_0]_t$. It follows from eq 1 that

$$\frac{[S]_t}{F_t} = \frac{[S]_0}{F_0} = \frac{[S_0]_0}{F_0} = \frac{1}{C'} \tag{3}$$

Combining eq 2 and 3 gives

$$v_t = -\frac{[S_0]_0 m_t}{F_0} = \frac{k_{cat}[E_0][S_0]_t}{K_M + [S_0]_t} \tag{4}$$

where $m_t = dF_t/dt$. Stopped-flow fluorescence traces defining F_t and m_t are shown in Figure 1. Letting $[S_0]_0 = [S_0]$, the reciprocal forms of eq 4 at times t and $t = 0$ (directly after mixing) are

$$-\frac{F_0[E_0]}{[S_0]m_t} = \frac{1}{k_{cat}} + \frac{K_M}{k_{cat}}\frac{1}{[S_0]_t} \tag{5}$$

$$-\frac{F_0[E_0]}{[S_0]m_0} = \frac{1}{k_{cat}} + \frac{K_M}{k_{cat}}\frac{1}{[S_0]} \tag{6}$$

Thus, one means of evaluating k_{cat} and K_M is from the m_0 and F_0 values obtained from a series of stopped-flow fluorescence traces obtained at variable $[S_0]$. Equation 6 predicts that a plot of $-F_0[E_0]/[S_0]m_0$ versus $1/[S_0]$ should be linear with x and y intercepts of $-1/K_M$ and $1/k_{cat}$, respectively. Alter-

natively, assuming that there is no P inhibition, these parameters can be evaluated from a single stopped-flow trace, since eq 5 predicts that a plot of $-F_0[E_0]/[S_0]m_t$ versus $1/[S_0]_t$ will give similar results. This procedure will produce accurate data only if $[S_0] > K_M$.

It should be noted that when $[S]_0 \ll K_M$, the $[S]_t$ term in the denominator of eq 2 is negligible. Using eq 3 to express $[S]_t$ in terms of F_t and integrating from 0 to t gives

$$F_t = F_0 e^{-(k_{cat}/K_M)[E_0]t} \tag{7}$$

Thus, k_{cat}/K_M can be determined from a single stopped-flow fluorescence trace obtained under conditions such that $[S_0] \ll K_M$ from a first-order plot.

An integrated form of eq 2 in which eq 3 has been used to replace $[S]_t$ by F_t is

$$\frac{F_0[E_0]t}{[S_0](F_0 - F_t)} = \frac{1}{k_{cat}} + \frac{K_M}{k_{cat}} \frac{F_0 \ln (F_0/F_t)}{[S_0](F_0 - F_t)} \tag{8}$$

This relationship is analogous to eq 10 of Orsi and Tipton (1979) and may be used to evaluate k_{cat} and K_M from a single stopped-flow trace carried out at a single concentration of S by plotting $F_0[E_0]t/[S_0](F_0 - F_t)$ versus $[F_0 \ln (F_0/F_t)]/[S_0](F_0 - F_t)$.

Solving eq 8 for t gives

$$t = \frac{[S_0](1 - F_t/F_0)}{k_{cat}[E_0]} + \frac{K_M \ln (F_0/F_t)}{k_{cat}[E_0]} \tag{9}$$

Integration of eq 9 gives

$$A_0 = \int_0^\infty F_t \, dt = \int_0^{F_0} t \, dF_t$$
$$= \int_0^{F_0} \left\{ \frac{[S_0](1 - F_t/F_0)}{k_{cat}[E_0]} + \frac{K_M \ln (F_0/F_t)}{k_{cat}[E_0]} \right\} dF_t \tag{10}$$

or

$$A_0 = \frac{F_0[S_0]}{2k_{cat}[E_0]} + \frac{K_M F_0}{k_{cat}[E_0]} \tag{11}$$

where A_0 is the total area under the observed fluorescence trace (Figure 1). Multiplying both sides of eq 11 by $[E_0]/F_0[S_0]$ gives

$$\frac{A_0[E_0]}{F_0[S_0]} = \frac{1}{2k_{cat}} + \frac{K_M}{k_{cat}} \frac{1}{[S_0]} \tag{12}$$

Thus, the kinetic parameters k_{cat} and K_M can also be determined from a family of fluorescence traces obtained at variable concentrations of S by plotting $A_0[E_0]/F_0[S_0]$ versus $1/[S_0]$.

In summary, the equations derived in this section show that the stopped-flow fluorescence traces for reactions in which F_t obeys eq 1 provide five different ways of evaluating the kinetic constants k_{cat}, K_M, or k_{cat}/K_M based on eq 5–8 and 12. These equations are similar in form, yet characteristically different, from those developed by Lobb and Auld (1984) to describe reactions in which F_t is proportional to the concentrations of ES_i.

Results

Preparation of [(LAP)Zn_6M_6] and Choice of Reaction Conditions. It has been shown in an earlier study (Lin & Van Wart, 1988b) that the fluorescence of both LAP and its dansyl substrates is quenched by Cu(II) and Ni(II). These ions have also been shown to slowly induce the degradation of Leu-Gly-NHNH-Dns. In contrast, Mg(II), Mn(II), and Zn(II) do not show these effects. In the past, [(LAP)Zn_6Cu_6] and [(LAP)Zn_6Ni_6] have been prepared by incubation of [(LAP)Zn_6—] with 0.1 mM solutions of Cu(II) and Ni(II), respectively, and subsequent experiments carried out in the presence of the same concentrations of these ions to keep the regulatory site saturated (Van Wart & Lin, 1981). However, since these ions adversely affect the fluorescence studies at these concentrations, a simple alternative procedure has been adopted.

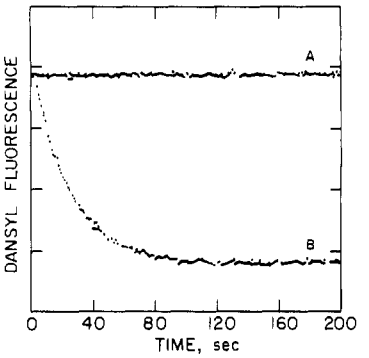

FIGURE 2: Stopped-flow fluorescence traces (λ_{ex} = 320 nm) for the mixing of 0.075 mM Leu-Gly-NHNH-Dns with 0.1 mM Cu(II) in 10 mM Tris and 0.1 M KCl, pH 9, with (A) 0.2 mM EDTA in both syringes and (B) no EDTA in either syringe.

First, [(LAP)Zn_6—] is incubated for 4 h in 10 mM Tris, pH 8 at 37 °C, containing 0.1 mM Ni(II) or Cu(II) to form the corresponding [(LAP)Zn_6M_6]. Next, EDTA (0.2 mM) is added to the enzyme (and substrate) solutions just prior to the stopped-flow experiment to chelate the free metal ions. The time dependence of the activities of several [(LAP)Zn_6M_6], where M(II) = Zn(II), Ni(II), and Cu(II), has been investigated after addition of 0.2 mM EDTA, as measured by Leu-*p*-nitroanilide assays (data not shown). Over a period of 2 h, there is a slow increase in activity that does not exceed 15%, confirming that any removal of regulatory metal ions by EDTA is slow. Thus, once [(LAP)Zn_6Ni_6] and [(LAP)-Zn_6Cu_6] have been formed, the addition of 0.2 mM EDTA does not have an appreciable effect on their activities over the time scale of the stopped-flow experiments. This is in agreement with earlier experiments that showed that the kinetics of exchange of Ni(II) and Cu(II) from the enzyme under these conditions is slow (Van Wart & Lin, 1981). The stopped-flow fluorescence experiments are then carried out as usual with the same concentration of EDTA in the enzyme and substrate solutions.

Stopped-flow fluorescence traces showing the mixing of Leu-Gly-NHNH-Dns with 0.1 mM Cu(II) when 0.2 mM EDTA was present and absent in both syringes are shown in Figure 2. The corresponding traces for Ni(II) are the same and confirm that the EDTA eliminates the changes in fluorescence due to complex formation and degradation reported elsewhere (Lin & Van Wart, 1988b). Since Mg(II), Mn(II), and Zn(II) do not exhibit the same adverse effects and identical stopped-flow fluorescence traces are obtained in the presence and absence of quantities of EDTA needed to chelate all excess ions, EDTA was not generally added in experiments with these ions. All reactions were carried out in 10 mM Tris and 0.1 M KCl, pH 9, on the basis of earlier results (Van Wart & Lin, 1981). Most of the reactions were carried out with Leu-Gly-NHNH-Dns, since the F_t changes are the largest for this substrate (Lin & Van Wart, 1988a). However, several experiments have been carried out with Leu-Gly-$NH(CH_2)_2$NH-Dns for comparison.

Analysis of Stopped-Flow Fluorescence Traces. Stopped-flow fluorescence experiments have been carried out to study the hydrolysis of Leu-Gly-NHNH-Dns by [(LAP)Zn_6M_6],

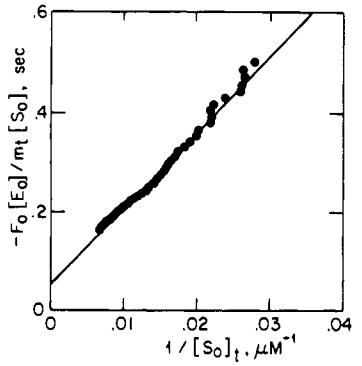

FIGURE 3: A plot of $-F_0[E_0]/m_t[S_0]$ versus $1/[S_0]_t$ from the reaction of 50 nM [(LAP)Zn_6Mg_6] with 200 μM Leu-Gly-NHNH-Dns in 10 mM Tris and 0.1 M KCl, pH 9 at 23 °C. The kinetic parameters calculated from this plot are k_{cat} = 1200 min^{-1} and K_M = 0.33 mM.

where M = Mg(II), Mn(II), Cu(II), Ni(II), Zn(II), and empty. All of these reactions are essentially irreversible, and there is no product inhibition by either Leu or Gly-NHNH-Dns (Lin & Van Wart, 1988a). In addition, the Gly-NHNH-Dns is not degraded further by the enzyme. Thus, the fluorescence traces have been analyzed by using the relationships derived under Theory in order to obtain the steady-state kinetic parameters for the reactions. The methods used to evaluate k_{cat} and K_M will be illustrated for the reaction of 50 nM [(LAP)Zn_6Mg_6] with variable concentrations (38–200 μM) of Leu-Gly-NHNH-Dns in 10 mM Tris and 0.1 M KCl, pH 9 (containing 5 mM $MgCl_2$ in both syringes).

Method 1 is based on eq 5, where $[S_0]_t$ is calculated from the following form of eq 3:

$$[S_0]_t = (F_t/F_0)[S_0] \quad (13)$$

The values of F_0 and F_t are obtained from each trace by subtracting the fluorescence intensity of the product, which corresponds to the flat portion of the trace at the end of the reaction, from that at times zero and t, respectively. To estimate the slope at time t, m_t, a suitable portion of the trace surrounding time point t is selected. The data collected during this interval are then fit to the exponential decay equation

$$F_t = Ae^{-kt} + B \quad (14)$$

and the slope at time t is calculated by differentiation. The slopes for the first several time points are estimated from the exponential fit to the interval covering approximately the first 40 points of the trace.

With this approach, a plot of $-F_0[E_0]/m_t[S_0]$ versus $1/[S_0]_t$ for the reaction of [(LAP)Zn_6Mg_6] with 200 μM Leu-Gly-NHNH-Dns has been constructed (Figure 3). Since LAP has six active sites per hexamer and k_{cat} is defined as the turnover number per active site, the values of $[E_0]$ used in this and all subsequent plots are the concentration of active sites. This number is sixfold higher than the concentration of hexamers given in the text and figure legends but allows k_{cat} for the hexameric enzyme to be obtained directly from the y intercepts of the kinetic plots. The plot shown in Figure 3 is linear and gives the kinetic parameters k_{cat} = 1200 min^{-1} and K_M = 0.33 mM. The maximum solubility of this substrate is 400 μM, which means that, after dilution to 200 μM in the stopped-flow experiment, the maximum $[S_0]$ is only $0.6K_M$. For the reactions in this study, this maximum solubility of S corresponds to between $0.54K_M$ and $2.5K_M$ (see below), indicating that the resulting parameters will have variable degrees of accuracy. When these reactions are carried out in 50% v/v methanol where the solubility of the substrate is much higher, this problem is eliminated (Lin et al., 1988). The inability to attain substrate solubilities severalfold higher than K_M is a problem for many LAP substrates (Taylor et al., 1981).

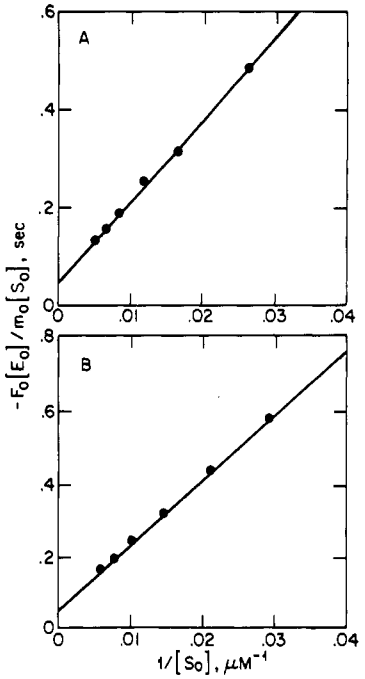

FIGURE 4: Plots of $-F_0[E_0]/m_0[S_0]$ versus $1/[S_0]$ for the reaction of 50 nM [(LAP)Zn_6Mg_6] with variable concentrations of Leu-Gly-NHNH-Dns in 10 mM Tris and 0.1 M KCl, pH 9 at 23 °C, constructed from (A) data obtained on a single time base to give k_{cat} = 1300 min^{-1} and K_M = 0.34 mM and (B) the data obtained with the initial, fast time base shown in Figure 5 to give k_{cat} = 1200 min^{-1} and K_M = 0.34 mM.

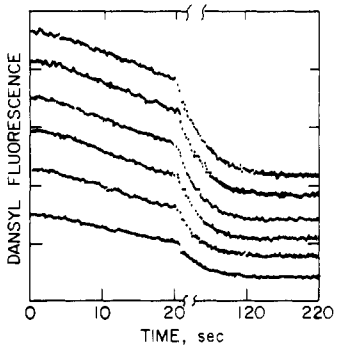

FIGURE 5: Stopped-flow fluorescence traces for the reaction of 50 nM [(LAP)Zn_6Mg_6] with variable concentrations of Leu-Gly-NHNH-Dns in 10 mM Tris and 0.1 M KCl, pH 9 at 23 °C, recorded on two different time bases. The substrate concentrations are (from top to bottom) 200, 150, 120, 85, 60, and 38 μM, respectively.

Methods 2 and 3 make use of a whole series of traces obtained at variable substrate concentrations and are based on eq 6. In method 2, m_0 is calculated from each trace from an exponential curve that is fit to the first 40 time points. A plot of $-F_0[E_0]/m_0[S_0]$ versus $1/[S_0]$ for the data is given in Figure 4A. This plot is linear and gives the kinetic parameters k_{cat} = 1300 min^{-1} and K_M = 0.34 mM. Method 3 involves the determination of m_0 from stopped-flow fluorescence traces recorded on two different time bases—an initial, very rapid time base that more clearly visualizes the initial fluorescence change, followed by a slower time base that allows determination of F_∞. A series of these traces for the reaction of 50 nM [(LAP)Zn_6Mg_6] with various concentrations of Leu-Gly-NHNH-Dns is shown in Figure 5. The fluorescence change recorded on the fast time scale is nearly linear with

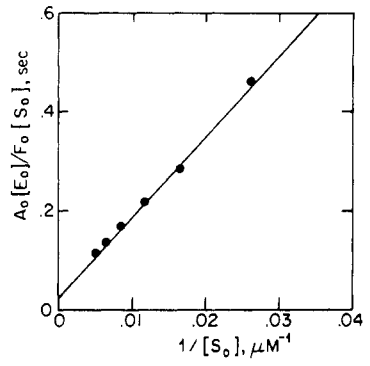

FIGURE 6: A plot of $A_0[E_0]/F_0[S_0]$ versus $1/[S_0]$ for the reaction of $[(LAP)Zn_6Mg_6]$ with Leu-Gly-NHNH-Dns in 10 mM Tris and 0.1 M KCl, pH 9 at 23 °C. The kinetic parameters calculated from this plot are k_{cat} = 1100 min^{-1} and K_M = 0.30 mM.

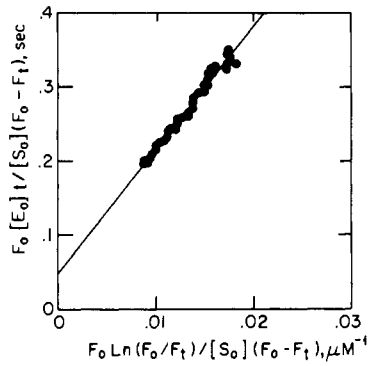

FIGURE 7: A plot of $F_0[E_0]t/[S_0](F_0 - F_t)$ versus $[F_0 \ln (F_0/F_t)]/[S_0](F_0 - F_t)$ constructed from the trace for the reaction of $[(LAP)Zn_6Mg_6]$ with 200 μM Leu-Gly-NHNH-Dns in 10 mM Tris and 0.1 M KCl, pH 9 at 23 °C. The kinetic parameters calculated from this plot and k_{cat} = 1300 min^{-1} and K_M = 0.36 mM.

time, and the estimate of m_0 is determined from a linear least-squares fit to this line. The second stage of the reaction recorded on the slower time base allows the determination of F_∞. A plot of $-F_0[E_0]/m_0[S_0]$ versus $1/[S_0]$ constructed from these traces is shown in Figure 4B. The plot is also linear and gives k_{cat} = 1200 min^{-1} and K_M = 0.34 mM.

Method 4 is based on eq 12, which uses the F_0 and A_0 values obtained from a series of stopped-flow traces obtained at different substrate concentrations. The total area under the trace, A_0, is obtained by summing all of the consecutive area elements, A_i, between data points. For any two consecutive time points, i and $i + 1$, A_i is approximated by $(1/2)(F_i + F_{i+1})\Delta T$. Since there are 200 time points for each trace, the time interval ΔT is very small and A_i is a good approximation to the actual area. A plot of $A_0[E_0]/F_0[S_0]$ versus $1/[S_0]$ for the reaction of $[(LAP)Zn_6Mg_6]$ with Leu-Gly-NHNH-Dns is illustrated in Figure 6 and gives k_{cat} = 1100 min^{-1} and K_M = 0.30 mM.

Method 5 is based on eq 8. The functions expressed on both sides of this equation are not defined at times $t = 0$ and ∞. Moreover, the values of $[\ln (F_0/F_t)]/(F_0 - F_t)$ are subject to substantial fluctuations due to experimental error in the vicinity of times 0 and ∞. Therefore, only data points covering the range of F_t/F_0 = 0.2–0.8 are used in the evaluation of the kinetic parameters. A plot of $F_0[E_0]t/[S_0](F_0 - F_t)$ versus $[F_0 \ln (F_0/F_t)]/[S_0](F_0 - F_t)$ for the reaction of $[(LAP)Zn_6Mg_6]$ with 200 μM Leu-Gly-NHNH-Dns is shown in Figure 7 and gives the kinetic parameters k_{cat} = 1300 min^{-1} and K_M = 0.36 mM.

Method 6 involves the determination of k_{cat}/K_M from the stopped-flow fluorescence trace of a reaction carried out when

Table I: Steady-State Kinetic Parameters for the Reaction of $[(LAP)Zn_6Mg_6]$ with Leu-Gly-NHNH-Dns Determined by Various Methods[a]

method	k_{cat} (min^{-1})	K_M (mM)	$(k_{cat}/K_M) \times 10^{-6}$ (min^{-1} M^{-1})
1	1200	0.33	3.6
2	1300	0.34	3.8
3	1200	0.34	3.5
4	1100	0.30	3.7
5	1300	0.36	3.6
6			3.8
mean	1200	0.33	3.6

[a] All reactions were carried out in 10 mM Tris and 0.1 M KCl, pH 9 at 23 °C.

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

enzyme	k_{cat} (min^{-1})	K_M (mM)	$(k_{cat}/K_M) \times 10^{-6}$ (min^{-1} M^{-1})
$[(LAP)Zn_6Mg_6]$	1200	0.33	3.6
$[(LAP)Zn_6Mn_6]$	1800	0.37	4.9
$[(LAP)Zn_6Ni_6]$	750	0.23	3.3
$[(LAP)Zn_6Cu_6]$	660	0.18	3.6
$[(LAP)Zn_6Zn_6]$	630	0.18	3.6
$[(LAP)Zn_6—]$	190	0.080	2.4

[a] All reactions were carried out in 10 mM Tris and 0.1 M KCl, pH 9 at 23 °C.

$[S]_0 \ll K_M$. Under these conditions, eq 7 is valid and can be written in the form

$$F_t = F_0 e^{-k_{obsd}t} \quad (15)$$

where $k_{obsd} = (k_{cat}/K_M)[E_0]$. Thus, the reaction is first order, and fitting the trace with an exponential gives k_{obsd}, which is used to calculate k_{cat}/K_M. The trace for the reaction of $[(LAP)Zn_6Mg_6]$ with 38 μM Leu-Gly-NHNH-Dns gives a value of k_{obsd} of 0.019 s^{-1}, from which k_{cat}/K_M is calculated to be 3.8 × 10^6 min^{-1} M^{-1}. A summary of the steady-state kinetic parameters k_{cat}, K_M, and k_{cat}/K_M for the hydrolysis of Leu-Gly-NHNH-Dns by $[(LAP)Zn_6Mg_6]$ obtained from the six different methods of analysis described above is given in Table I. The agreement for the values of these parameters obtained by the different methods is excellent.

Determination of Kinetic Parameters for Other $[(LAP)Zn_6M_6]$. The stopped-flow fluorescence traces for the reactions of Leu-Gly-NHNH-Dns with $[(LAP)Zn_6M_6]$ where M(II) = Mn(II), Ni(II), Cu(II), Zn(II), and empty are all qualitatively similar to those for $[(LAP)Zn_6Mg_6]$ and exhibit a single hyperbolic relaxation. The same kinetic analyses described above for $[(LAP)Zn_6Mg_6]$ have been applied to determine the steady-state kinetic parameters for these enzymatic reactions. All of the plots were linear with good fits, and the different methods gave good agreement. The average values of the kinetic parameters obtained by the six different methods are summarized in Table II. It can be seen that the identity of the metal ion in the regulatory site influences both k_{cat} and K_M. The largest effect is seen for Mn(II), which raises k_{cat} 9.5-fold and K_M 4.6-fold. In fact, all of the M(II) raise both k_{cat} and K_M, with the result that k_{cat}/K_M only varies by approximately twofold. This differs from the results found when Leu-*p*-nitroanilide is used as substrate which indicate that the regulatory M(II) only affects k_{cat} (Van Wart & Lin, 1981).

The hydrolysis of Leu-Gly-$NH(CH_2)_2$NH-Dns by $[(LAP)Zn_6Mg_6]$ was also investigated for comparison. The stopped-flow fluorescence traces for this reaction are very similar to those for Leu-Gly-NHNH-Dns, except that the F_t values *increase* with time and are somewhat noisier due to the

smaller magnitude of F_0 (Lin & Van Wart, 1988a). However, all methods of analysis are essentially the same except that the values of m_t are positive and those of F_t are negative. The kinetic parameters for this reaction are k_{cat} = 930 min^{-1} and K_M = 0.32 mM and are very similar to those for Leu-Gly-NHNH-Dns.

Discussion

It was established in the preceding paper (Lin & Van Wart, 1988a) that the stopped-flow fluorescence changes, F_t, observed for the reaction of [(LAP)Zn_6M_6] with its dansyl substrates arise solely from variations in the concentrations of substrate and product during the reaction. In order to abstract information about the steady-state kinetics of these reactions, a series of relationships have been derived which permit the parameters k_{cat}, K_M, and k_{cat}/K_M to be evaluated from measurements of the values of F_t, A_t, and m_t, or F_0, A_0, and m_0, from the stopped-flow traces. These relationships are similar to, yet different from, those derived by Lobb and Auld (1984) for systems in which there is energy transfer between enzyme and dansyl substrate. In such systems, the F_t are proportional to the concentrations of ES_i and the measurement of F_t and A_t, or F_0 and A_0, values only is employed for the analyses. The agreement between the values of k_{cat} and K_M calculated by the six methods described herein is excellent and allows a wide choice of data analysis methods. Moreover, reactions like those described in this paper that exhibit fluorescence changes that arise from changes in the free substrate concentration can be more easily designed than those that exhibit energy transfer and offer an alternative for enzyme–substrate systems not amenable to the energy-transfer approach.

It is of interest to compare the kinetic parameters listed in Table II for the hydrolysis of Leu-Gly-NHNH-Dns by the various [(LAP)Zn_6M_6] with those for Leu-*p*-nitroanilide that have been determined previously (Lin & Van Wart, 1981). First, for a given [(LAP)Zn_6M_6], the value of k_{cat}/K_M for the hydrolysis of Leu-Gly-NHNH-Dns is considerably larger than that for Leu-*p*-nitroanilide, ranging from 86-fold higher for [(LAP)Zn_6Mg_6] to 1800-fold higher for [(LAP)Zn_6Zn_6]. This is due predominantly to higher values of k_{cat} that range from 29- to 315-fold higher for [(LAP)Zn_6Mg_6] and [(LAP)-Zn_6Zn_6], respectively. However, the rise in k_{cat}/K_M is also partly due to values of K_M that decrease from 12.5-fold for [(LAP)Zn_6—] to 2.7-fold for [(LAP)Zn_6Mn_6]. Since the kinetic parameters for the hydrolysis of Leu-Gly-NHNH-Dns and Leu-Gly-NH$(CH_2)_2$NH-Dns by [(LAP)Zn_6Mg_6] are very similar, both are probably better substrates for LAP than Leu-*p*-nitroanilide because they contain a Gly residue on the C-terminal side of the scissile bond, allowing for the $P'_1 \cdots S'_1$ interactions present for the natural protein and peptide substrates of the enzyme.

The effect of the regulatory metal ion on the k_{cat} and K_M values for these two substrates is also quite different. For Leu-*p*-nitroanilide, Mg(II) and Mn(II) raise k_{cat} and Ni(II), Cu(II), and Zn(II) lower k_{cat}, while K_M remains almost unchanged for all of these metal ions (Van Wart & Lin, 1981). In contrast, for Leu-Gly-NHNH-Dns, these metal ions all raise k_{cat}, ranging from 3.3-fold for Zn(II) to 9.5-fold for Mn(II). However, K_M is also raised by all of these metal ions from 2.3-fold for Cu(II) and Zn(II) to 4.6-fold for Mn(II), so that the values of k_{cat}/K_M are only increased slightly. Thus, if k_{cat}/K_M is used as a criterion, [(LAP)Zn_6—] is maximally activated by only approximately twofold by any of these metal ions and the difference between the activities for the five metallohybrids is only 1.5-fold. This contrasts sharply with the results for Leu-*p*-nitroanilide, where the k_{cat}/K_M values of [(LAP)Zn_6M_6] are significantly higher for Mg(II) and Mn(II) and slightly lower for Ni(II), Cu(II), and Zn(II) compared to [(LAP)Zn_6—]. Thus, the effect of the metal ion in the regulatory site on all three parameters k_{cat}, K_M, and k_{cat}/K_M is markedly dependent on the substrate.

In view of these results, it is appropriate to reexamine earlier conclusions concerning the role of the regulatory metal ion in influencing the activity of porcine kidney LAP. The data presented here suggest that conclusions derived from the use of substrates that lack an amino acid residue in subsite P'_1 can be misleading. Any conclusion pertaining to the physiological significance of the regulatory metal ion on the activity of LAP will clearly depend upon the criterion for activity that is adopted. In particular, if LAP-catalyzed reactions in vivo are assumed to take place under conditions that are zero order in substrate ($[S_0] \gg K_M$), then k_{cat} is the important parameter. Alternatively, if the reactions take place under conditions that are first order in substrate ($[S_0] \ll K_M$), the k_{cat}/K_M reflects the relative activities.

Assuming that the results for Leu-Gly-NHNH-Dns shown in Table II accurately reflect those for a physiological substrate, all five of the M(II) can legitimately be claimed to activate the enzyme under zero-order conditions and the term "activation site" is justified. However, if the reaction occurs in vivo under first-order conditions, the effect of these metal ions on activity is very small and it is questionable whether the differences in rate are of any significance. In this case, these five "regulatory" M(II) have essentially no influence on the physiological activity of the enzyme and the terms "regulatory" and "activation" are misnomers. Thus, the possibility exists that the marked stimulation of porcine kidney LAP by Mg(II) and Mn(II) is a misleading laboratory observation attributable to the use of nonphysiological substrates or of specific substrates assayed under nonphysiological kinetic conditions. Since most previous kinetic studies of bovine lens LAP metallohybrids have also employed substrates lacking an amino acid in subsite P'_1 (Carpenter & Vahl, 1973; Thompson & Carpenter, 1976a,b; Allen et al., 1983), the conclusions of these studies pertaining to the effects of the metal ion at the two different binding sites on activity may likewise require reinterpretation. The influence of the regulatory metal ions on the pre-steady-state kinetics of hydrolysis of Leu-Gly-NHNH-Dns by LAP is considered in the following paper (Lin et al., 1988).

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Registry No. LAP, 9001-61-0; Leu-Gly-NHNH-Dns, 89315-19-5; Leu-Gly-NH$(CH_2)_2$NH-Dns, 114333-74-3.

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

Wann-Yin Lin, Spencer H. Lin, Roger J. Morris, and Harold E. Van Wart*
Department of Chemistry and Institute of Molecular Biophysics, Florida State University, Tallahassee, Florida 32306
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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

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* Author to whom correspondence should be addressed at the Institute of Molecular Biophysics.

[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.

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