Hydrolysis of Dansyl-Peptide Substrates by Leucine Aminopeptidase: Origin of Dansyl Fluorescence Changes during Hydrolysis†

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ABSTRACT: The origin of the fluorescence changes observed in stopped-flow experiments of the hydrolysis of three 5-(dimethylamino)naphthalene-1-sulfonyl-(dansyl) peptide substrates by porcine kidney cytosol leucine aminopeptidase has been investigated. The substrates used all have the potential to accept energy from aromatic residues of the enzyme via resonance energy transfer when they are bound as enzyme–substrate complexes, indicating that fluorescence changes due to the buildup and decay of such intermediates are possible. However, the fluorescence of these substrates differs from that of the products, and direct excitation of their dansyl groups during hydrolysis can also be responsible for the observed fluorescence changes due to changes in the concentrations of free substrate and product. The dansyl fluorescence changes observed with excitation wavelengths near 280 nm are not accompanied by quenching of the enzyme fluorescence, as would be expected if there were enzyme-to-substrate energy transfer. The magnitude of the maximal fluorescence change at a fixed concentration of substrate is also independent of the enzyme concentration. Furthermore, the excitation profile for the fluorescence changes shows that they arise from direct excitation of the dansyl group. Thus, there is no energy transfer in these reactions, and the fluorescence changes observed arise from direct excitation of the dansyl group and reflect the instantaneous concentration of substrate. This behavior contrasts sharply with that for the reaction of carboxypeptidase A with dansyl-Gly-Tyr, which has been studied as a positive control for an energy-transfer system. The failure to observe energy transfer between leucine aminopeptidase and its dansyl substrates during hydrolysis may be due to alterations in the spectral properties of the substrates on binding to the enzyme.

Porcine kidney cytosol leucine aminopeptidase (LAP)[1] (EC 3.4.11.1) is a hexameric metalloenzyme that hydrolyzes the N-terminal peptide bond to most amino acids (Himmelhoch, 1970; Delange & Smith, 1971). Each subunit contains a catalytic and a regulatory metal binding site (Van Wart & Lin, 1981). In the absence of activating metal ions, the catalytic site is occupied by Zn(II) and the regulatory site is vacant, and the enzyme is represented as $[(LAP)Zn_6—]$. On incubation with various divalent metal ions, M(II), the regulatory site becomes occupied to form a metallo hybrid, denoted $[(LAP)Zn_6M_6]$. Depending upon the identity of M(II), this binding is accompanied by an increase or decrease in activity toward Leu-*p*-nitroanilide (Van Wart & Lin, 1981). In this paper and the following papers (Lin et al., 1988a,b), stopped-flow fluorescence studies have been carried out to study the kinetics of hydrolysis of dansyl-peptide substrates by several $[(LAP)Zn_6M_6]$.

Stopped-flow studies of enzyme-catalyzed reactions permit the study of both the steady-state and pre-steady-state kinetic regimes (Auld, 1977). Steady-state kinetic studies allow the measurement of the macroscopic kinetic parameters k_{cat} and K_M that describe all enzymatic reactions. In addition, the stopped-flow technique allows one to examine the pre-steady-state reaction with the opportunity to resolve elementary steps in the catalytic pathway. For example, it is possible to observe directly transient enzyme–substrate intermediates (ES_i) formed during the reaction and to determine the values of the microscopic rate constants that describe their formation and decay (Gibson, 1969; Chance, 1974; Auld, 1977), provided that a means of visualizing such intermediates is in hand. Fluorescent peptide substrates have been used successfully with proteinases to permit the detection of ES_i and quantitation of their rates of interconversion. Such studies use either direct fluorophore excitation (Sachdev & Fruton, 1975; Mattis & Fruton, 1976; Morgan & Fruton, 1978) or excitation of the enzyme followed by radiationless energy transfer to the substrate (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) to observe the ES_i.

To be able to abstract meaningful kinetic information from the fluorescence changes observed during either the steady-state or pre-steady-state regimes in stopped-flow experiments, it is first necessary to establish the origin of the fluorescence changes. This is because the kinetic formalism used to treat these fluorescence changes depends upon their source. The steady-state kinetic studies to be described here were carried out under the conditions $[S_0] \gg [E_0]$. Under these conditions, the fluorescence changes for enzyme–substrate systems in which enzyme-to-substrate energy transfer (ET) produces all of the observed fluorescence reflect solely the concentrations of ES_i. Alternatively, in systems in which the fluorescence

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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; ET, energy transfer; DE, direct excitation; E, enzyme; S, substrate; P, product; ES, enzyme–substrate intermediate.

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arises from direct excitation (DE) of the dansyl group, the changes can reflect solely the concentration of free substrate. In this study, the origin of the fluorescence changes observed on hydrolysis of dansyl-peptides by various [(LAP)Zn_6M_6] has been thoroughly investigated. It is shown that, although this enzyme–substrate system satisfies the criteria necessary for radiationless ET, the changes in dansyl fluorescence observed in the steady-state stopped-flow experiments are due to changes in the concentration of the dansyl substrate. In the second of three papers in this issue (Lin et al., 1988b), this information is used to analyze the stopped-flow data and obtain the desired steady-state kinetic parameters. In the third of three papers in this issue (Lin et al., 1988a), the pre-steady-state reaction of these [(LAP)Zn_6M_6] with a dansyl substrate is studied by DE of the ES_i.

Materials and Methods

Materials. LAP was obtained from Sigma Chemical Co. (type III-CP) or Accurate Chemical and Science Co. as a suspension in 2.9 M $(NH_4)_2SO_4$ and 0.1 M Tris containing 5 mM $MgCl_2$ and purified by affinity chromatography over Leu-Gly-AH-Sepharose (Van Wart & Lin, 1981). AH-Sepharose was a product of Pharmacia, carboxypeptidase A was obtained from Sigma Chemical Co., and Chelex 100 resin was obtained from Bio-Rad. $MgCl_2$, $MnSO_4$, $CuSO_4$, $NiCl_2$, and $ZnSO_4$ were spectrographically pure salts from Johnson-Matthey Chemicals Ltd. Leu-Gly-NHNH-Dns, Leu-Gly-$NH(CH_2)_2$NH-Dns, Leu-Gly-$NH(CH_2)_6$NH-Dns, and Dns-Gly-Tyr were synthesized as described by Lin and Van Wart (1988).

Metal-Free Procedures. Since LAP is a metalloenzyme whose activity is affected by many metal ions, all experimental protocols were carried out under metal-free conditions and extreme care was taken to prevent contamination from adventitious metal ions. Failure to do so may cause marked alterations in enzyme activity and erroneous kinetic measurements. Therefore, reagent-grade water with a resistivity of 18 MΩ/cm was prepared with a Millipore Milli-Q system. All buffer and salt solutions were rendered metal free with Chelex 100 resin, and solutions of all substrates were extracted with dithizone in carbon tetrachloride. Dialysis tubing was cleaned by repeated washing in 60–70 °C metal-free water. Only plasticware was used, except for quartz cuvettes which were rendered metal free by soaking them in dilute metal-free HCl. Solutions of metal ions were prepared from Johnson-Matthey spectrographically pure salts.

Routine Absorption and Fluorescence Measurements. The absorption spectra were obtained with a Varian Model 219 spectrophotometer, and the excitation and emission spectra were recorded with a Perkin-Elmer Model LS-5 fluorometer. The concentrations of LAP hexamers and all dansyl substrates were determined spectrophotometrically by using $\epsilon_{280} = 4.0 \times 10^5$ M^{-1} cm^{-1} and $\epsilon_{330} = 4.5 \times 10^3$ M^{-1} cm^{-1}, respectively.

Steady-State Assays. Assays for the hydrolysis of Leu-*p*-nitroanilide in the presence of Leu and Gly-NHNH-Dns were carried out as described in Lin and Van Wart (1988).

Stopped-Flow Fluorescence Studies. The stopped-flow experiments were carried out with an instrument described elsewhere (Van Wart & Zimmer, 1981). Monochromatic light is produced with an Oriel-150W xenon lamp and an Instruments SA, Inc., Model H10 monochromator and focused onto the observation cell of the stopped-flow instrument with either a quartz lens or a liquid light guide. The light emitted perpendicular to the incident beam is passed through an Oriel 420-nm long-pass filter to select the dansyl emission or a 350-nm band-pass filter to select the enzyme emission and carried via a liquid light guide to a Hamamatsu Model R136 photomultiplier tube. The signal was displayed and recorded on an Aminco DASAR (Data Acquisition, Storage, and Retrieval). The data were then transferred to an Apple IIe computer using an Interactive Microware ADA–AMP and stored on a floppy disk for later analysis. The preparation of the [(LAP)Zn_6M_6] samples and other details of the stopped-flow experiments are given in the following paper (Lin et al., 1988b), in which the stopped-flow traces are analyzed in detail.

Theory

Before considering the stopped-flow fluorescence traces for the reaction of LAP with its dansyl substrates, it will be useful to review the kinetic equations that describe the changes for such reactions. Depending on the choice of enzyme, substrate, and reaction conditions, the source of the observed fluorescence changes can vary. In particular, it will become essential to distinguish between systems in which the fluorescence changes are due solely to variations in the concentrations of substrate (S) and product (P) versus those in which the changes are due solely to variations in the concentrations of ES_i.

Consider the following general reaction scheme in which enzyme (E) catalyzes the irreversible conversion of S to P without product inhibition or activation:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES_1 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \cdots \rightleftharpoons ES_i \underset{k_{-(i+1)}}{\overset{k_{(i+1)}}{\rightleftharpoons}} \cdots \rightleftharpoons ES_n \underset{k_{-(n+1)}}{\overset{k_{(n+1)}}{\rightarrow}} E + P \quad (1)$$

The change in dansyl fluorescence observed in a stopped-flow fluorescence experiment at time t, F_t, is potentially related to the instantaneous concentrations of all of the dansyl species. A general expression for F_t (that is valid as long as the fluorophore concentration is sufficiently low that it is proportional to its fluorescence) follows from the analysis of Lobb and Auld (1984)

$$F_t = C(Q_S a_S - Q_P a_P)([S_0] - [P]_t) + F^{DE} + F^{ET} \quad (2)$$

where Q_S and a_S are the quantum yields and molar absorptivities, respectively, of the dansyl group of S at the excitation wavelength, Q_P and a_P are the same for P, C is a constant, $[S_0]$ is the initial total substrate concentration, $[P]_t$ is the free product concentration at time t, and F^{DE} and F^{ET} are the contributions to F_t due to the ES_i that arise via their DE or from ET, respectively. Both of the latter quantities exhibit a dependence on the concentration of the ES_i as shown (Lobb & Auld, 1984)

$$F^{DE} = C\sum(Q_i a_i - Q_S a_S)[ES_i] \quad (3)$$

$$F^{ET} = I_0 C_D \sum Q_i a_i T_i [ES_i] \quad (4)$$

where I_0 is the incident light intensity, T_i is the efficiency of ET in ES_i, and C_D is an instrumental factor. There is no simple relationship between the parameters measured in stopped-flow fluorescence experiments (see below) and the Michaelis–Menten parameters k_{cat} and K_M when all of the terms in eq 2 are nonnegligible. However, by proper choice of the enzyme and dansyl substrate and the use of appropriate experimental conditions, three types of situations can be distinguished which correspond to those in which only one of the three terms in eq 2 is nonnegligible.

Energy-Transfer Experiments. Stopped-flow fluorescence ET experiments are carried out under conditions such that the first two terms in eq 2 are negligible. The magnitude of the first term is minimized by utilizing substrates for which $Q_S a_S$ and $Q_P a_P$ are nearly identical. The second term is minimized

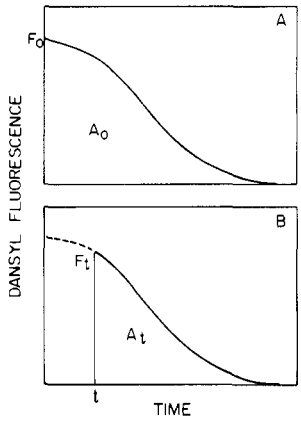

FIGURE 1: Schematic illustration of a stopped-flow fluorescence trace showing the change of dansyl fluorescence with time and defining the parameters (A) F_0 and A_0 and (B) F_t and A_t.

by employing excitation wavelengths that are distant from those of S and choosing conditions where $[S_0] \gg [E_0]$, so that little or no fluorescence due to DE of ES_i is expected. Thus, in stopped-flow fluorescence ET experiments, the fluorescence change observed during the reaction is due entirely to the buildup and decay of the ES_i. Typical stopped-flow fluorescence ET oscilloscope traces are shown in Figure 1 to define the important parameters for a reaction carried out at a fixed concentration of S and E. F_t is the magnitude of the fluorescence change at time t after initiation of the reaction, and F_0 is F_t at $t = 0$. A_t is the area under the curve from time t until completion of the reaction, while A_0 is the total area under the curve.

The fluorescence changes that are observed in ET experiments have very characteristic behavior. First, eq 4 predicts that F^{ET} for dansyl fluorescence can only be positive, since none of the terms on the right-hand side of the equation can be less than zero. Accordingly, F_t as defined in Figure 1 is positive and nonzero until the reaction is complete. Second, since $[ES_i]$ is proportional to $[E_0]$ when $[E_0] \gg [S_0]$, it follows from eq 4 that F_0 is proportional to $[E_0]$. Third, in a stopped-flow experiment in which $[S_0] \gg K_M$, the trace of F_t versus time will have a sigmoid shape, since the concentrations of ES_i change slowly at the start, but then more rapidly as $[S_0]$ is lowered to the vicinity of K_M. Fourth, a stopped-flow trace of the fluorescence of E should show a mirror image quenching (decrease in the F_t of E), followed by the return of fluorescence with time. Fifth, a stopped-flow fluorescence excitation profile of F_0 should have a maximum that corresponds to the absorbance of the aromatic donor residues on E.

Direct Excitation of ES_i Only. Fluorescence changes due to variations in the concentration of ES_i can also be studied by exciting the ES_i directly. In DE experiments, a dansyl substrate is chosen for which $Q_S a_S = Q_P a_P$ to eliminate the first term in eq 2. The F^{DE} term is enhanced by carrying out the reaction under conditions where $[E_0] \gg [S_0]$ and by employing excitation wavelengths within the absorption band of the ES_i, but away from E and, if possible, away from S. The characteristics of such reactions are somewhat different from those for ET experiments. Specifically, eq 3 predicts that F_t can be either positive or negative, depending on the relative values of $Q_i a_i$ and $Q_S a_S$ at the excitation wavelength. Another difference is that, since the fluorescence of the ES_i arises from DE, there is no corresponding quenching of the fluorescence of E. Moreover, the stopped-flow fluorescence excitation profile should mirror the absorption spectrum of S in the ES_i rather than that of S or E. As with the ET experiments, however, F_0 will be proportional to $[E_0]$ and the shape of the stopped-flow fluorescence trace will be sigmoid in shape when $[S_0] \gg K_M$.

Direct Excitation of S and P Only. When the stopped-flow fluorescence experiments are carried out with a S and at a wavelength at which $Q_S a_S$ does not equal $Q_P a_P$, the first term in eq 2 is nonzero and, in fact, can be quite large. If the excitation wavelength is distant from the absorbance of the enzyme, there will be no enzyme-to-substrate ET and the F^{ET} term in eq 2 can be neglected. Furthermore, as long as $[S_0] \gg [E_0]$, the concentration of ES_i will be very small compared to [S] and the last term in eq 2 is also negligible. Thus, the fluorescence observed is due to S and P only. Recognizing that $[S_0] - [P]_t = [S]_t$ under these conditions, eq 2 takes the form

$$F_t = C(Q_S a_S - Q_P a_P)[S]_t \quad (5)$$

where $[S]_t$ is the concentration of free S at time t. Since $C(Q_S a_S - Q_P a_P)$ is a constant in any given experiment, F_t simply reflects the instantaneous concentration of free S.

With this in mind, the fluorescence changes in experiments involving DE of S have a third distinct set of characteristics. First, like DE experiments with ES_i, F_t can be either positive or negative, since this is determined by the relative values of $Q_S a_S$ and $Q_P a_P$. Another similarity is that the dansyl F_t changes are not mirrored by those of E. These experiments differ from the previous two in that F_0 is independent of $[E_0]$. In these experiments, the slope of the stopped-flow fluorescence trace at time t is proportional to the instantaneous rate. Since this rate can only decrease with time in these systems, the shape of the stopped-flow trace is hyperbolic and cannot be sigmoid, as shown in Figure 1. Last, the stopped-flow fluorescence profile for F_t should correspond to the difference between the excitation spectra of S and P.

Results and Discussion

The objective of the experiments described in this paper is to establish the basis for the fluorescence changes observed in stopped-flow experiments on the hydrolysis of dansyl substrates by $[(LAP)Zn_6M_6]$. This is a necessary prerequisite to using these stopped-flow traces to determine the steady-state kinetic parameters for these reactions. The three substrates under study are Leu-Gly-NHNH-Dns, Leu-Gly-NH-$(CH_2)_2$NH-Dns, and Leu-Gly-NH$(CH_2)_6$NH-Dns, all of which have the free amino terminus and appropriate amino acid residues in subsites P_1 and P_1' [nomenclature of Schechter and Berger (1967)] necessary to make them LAP substrates. Details pertaining to the preparation of the $[(LAP)Zn_6M_6]$ and the choice of reaction conditions are deferred to the following paper (Lin et al., 1988b), in which the steady-state kinetics of these reactions are analyzed. A series of experiments has also been carried out with carboxypeptidase A and Dns-Gly-Tyr. This enzyme–substrate system, which has spectral properties very similar to those of LAP and its dansyl substrates, is shown to undergo ET. Thus, it will serve as a positive control for the behavior expected for ET systems.

Spectral Properties of Enzymes and Substrates. The absorption and emission bands of both carboxypeptidase A and LAP and their respective dansyl substrates satisfy the necessary criteria for ET studies. The excitation and emission spectra of carboxypeptidase A and LAP are very similar (Figure 2). Both enzymes have an emission band centered near 330–335 nm that arises from excitation of aromatic (presumably Trp) residues of the enzyme near 277 nm. All four dansyl substrates have similar absorption and emission spectra with an absorption band near 320–328 nm and an emission band (λ_{ex} = 320 nm) near 520–560 nm (Figure 3). Dns-Gly-Tyr also has

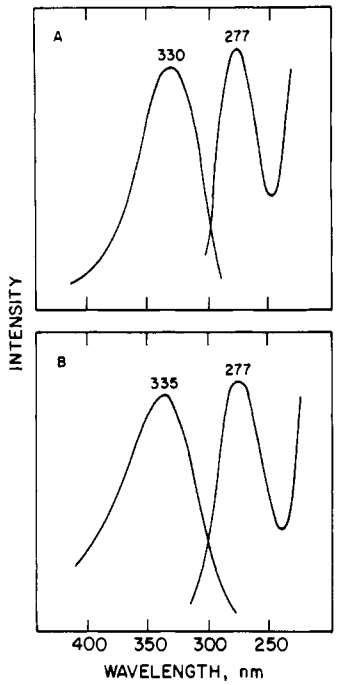

FIGURE 2: Excitation and emission spectra of (A) 5 μM carboxypeptidase A (λ_{em} = 330 nm, λ_{ex} = 280 nm) in 50 mM Tris and 1 M NaCl, pH 7.5, and (B) 250 nM [(LAP)Zn_6Mg_6] (λ_{em} = 335 nm; λ_{ex} = 277 nm) in 50 mM Tris and 0.1 M KCl, pH 9 at 23 °C.

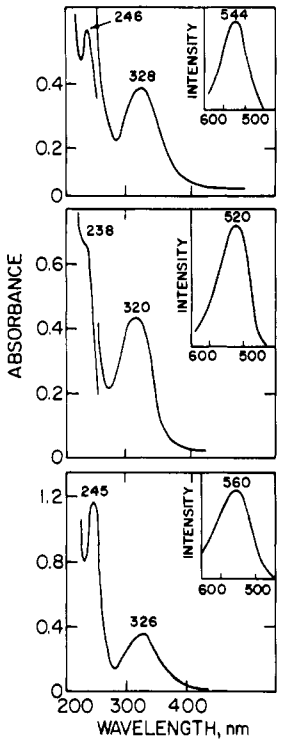

FIGURE 3: Absorption and (inserts) emission (λ_{ex} = 330 nm) spectra of (top) Dns-Gly-Tyr in 50 mM Tris and 1 M NaCl, pH 7.5, (middle) Leu-Gly-NHNH-Dns, and (bottom) Leu-Gly-$NH(CH_2)_nNH$-Dns (same spectra for n = 2, 6) in 10 mM Tris and 0.1 M KCl, pH 9 at 23 °C.

a weak Tyr emission band (not shown) centered at 313 nm (λ_{ex} = 270 nm) which overlaps its own dansyl absorption band, thus leading to some internal Tyr-to-dansyl ET. However, this transfer is almost negligible when the excitation wavelength is shifted to 285 nm or greater. For both enzymes, the emission band near 325 nm overlaps the absorption band of its dansyl substrate(s), indicating that each pair constitutes a potential ET system in which excitation of E near 277 nm can potentially lead to ET in the ES_i to produce dansyl fluorescence near 520–560 nm. Indeed, such ET has already been demonstrated for carboxypeptidase with similar dansyl substrates (Latt et al., 1970; Auld et al., 1972; Auld & Holmquist, 1974; Auld, 1977; Galdes et al., 1983, 1986; Williams & Auld, 1986).

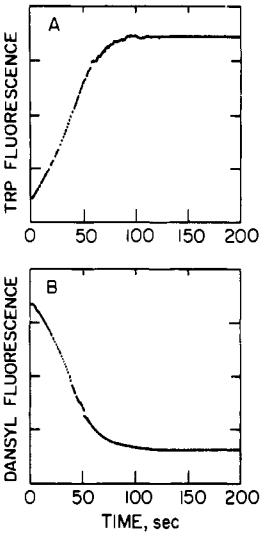

FIGURE 4: Stopped-flow fluorescence traces (λ_{ex} = 285 nm) showing the changes in (A) Trp and (B) dansyl fluorescence during the hydrolysis of 84 μM Dns-Gly-Tyr by 12 μM carboxypeptidase A in 50 mM Tris and 1 M NaCl, pH 7.5 at 23 °C.

Stopped-Flow Experiments with Carboxypeptidase A. First, the stopped-flow fluorescence traces for the hydrolysis of Dns-Gly-Tyr by carboxypeptidase A are considered. This reaction has been carried out under conditions designed to allow the F^{ET} term in eq 2 to dominate. First, the absorption and emission spectra of Dns-Gly-Tyr (S) and Dns-Gly (P) when excited at 285 nm are almost identical (data not shown). Thus, the stopped-flow fluorescence traces for the hydrolysis of Dns-Gly-Tyr obtained with excitation at 285 nm should not contain any contribution from the first term in eq 2, since $Q_S a_S = Q_P a_P$. There is also no *internal* ET between the Tyr and Dns groups of S when excitation at 285 nm is employed (see below). Second, the contribution to the fluorescence changes from the F^{DE} term (eq 3) is negligible, since the excitation wavelength is far from the absorption band of the dansyl group. Thus, the fluorescence changes observed for this reaction on excitation at 285 nm are expected to be due to ET in the ES_i only.

Stopped-flow traces showing the changes in both Trp and dansyl fluorescence that occur during the reaction of carboxypeptidase A with Dns-Gly-Tyr in 50 mM Tris and 1 M NaCl, pH 7.5, at 23 °C are shown in Figure 4. The first event after mixing is the combination of E and S within the mixing time of the instrument to form one or more ES_i. This procedure produces an instantaneous increase in dansyl fluorescence and corresponding decrease in Trp emission. Next, the ES_i decay slowly to form P and regenerate free E, as reflected by the slow decrease in dansyl fluorescence and return of Trp emission. The parallel, but opposite, changes in the Trp and dansyl emission traces are clear evidence that the changes in F_t arise from the F^{ET} term.

The shape of the dansyl stopped-flow fluorescence trace shown in Figure 4B is characteristic of those found in ET studies whenever $[S_0] > K_M$. The values of $[S_0]$ and K_M for this reaction are 84 and 36 μM, respectively. Under these circumstances, 70% of E is initially complexed with S to form ES_i, whose concentrations decay slowly until $[S_0]$ becomes similar in value to K_M. At this point, the ES_i decay more

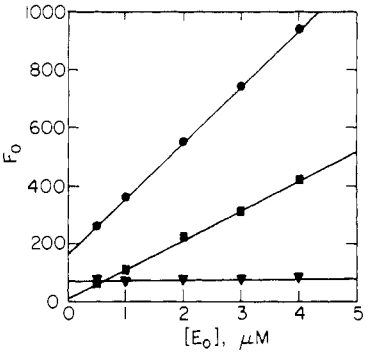

FIGURE 5: Dependence of F_0 on [E_0] for the reaction of carboxypeptidase A with 0.1 mM Dns-Gly-Tyr in 50 mM Tris and 1 M NaCl, pH 7.5 at 23 °C, for excitation wavelengths of (●) 280, (■) 290, and (▼) 330 nm.

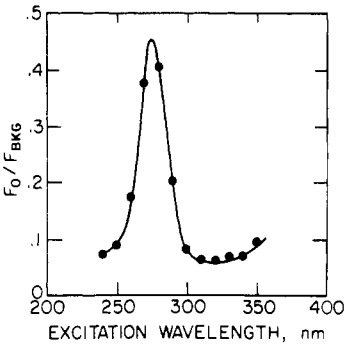

FIGURE 6: Excitation profile for the maximal change in dansyl fluorescence (F_0) relative to the fluorescence background (F_{bkg}) for the reaction of 5 μM carboxypeptidase A with 0.1 mM Dns-Gly-Tyr in 50 mM Tris and 1 M NaCl, pH 7.5 at 23 °C, obtained from stopped-flow experiments.

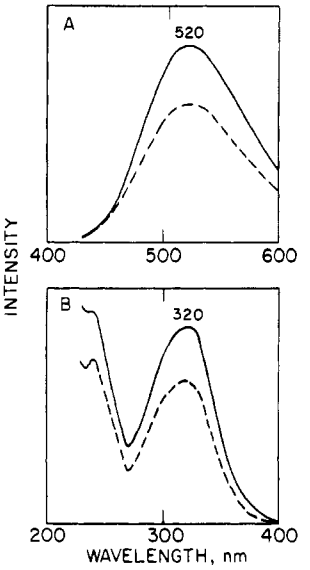

FIGURE 7: (A) Emission (λ_{ex} = 320 nm) and (B) excitation (λ_{em} = 520 nm) spectra of 0.1 mM Leu-Gly-NHNH-Dns in 50 mM Tris and 0.1 M KCl, pH 9 at 23 °C, (—) before and (---) after hydrolysis.

rapidly and eventually go to zero as [S_0] approaches zero.

A characteristic feature of ET systems is that F_0 is directly proportional to [E_0] as long as [S_0] $\gg$ [E_0]. The F_0 values for the hydrolysis of Dns-Gly-Tyr by carboxypeptidase A as a function of [E_0] have been measured at a fixed [S_0] of 0.1 mM. Plots of F_0 versus [E_0] for excitation wavelengths of 280, 290, and 330 nm are shown in Figure 5. The values of F_0 obtained by DE of substrate at 330 nm are small and independent of [E_0]. This is expected since there can be almost no ET under these circumstances. Any fluorescence changes observed at this wavelength would be due to either the first or second terms of eq 2. In contrast, the plot for excitation at 280 nm clearly shows that F_0 varies linearly with [E_0], as expected when there is ET in the ES_i. The nonzero intercept on the F_0 axis is due to some Tyr-to-Dns internal ET in Dns-Gly-Tyr that is lost on hydrolysis and whose magnitude depends only on [S_0]. This contribution from the internal ET of S is eliminated by exciting the system at a slightly longer wavelength where the Tyr absorbance is almost zero. Thus, the values of F_0 obtained by using excitation at 290 nm were found to be directly proportional to [E_0] with *no contribution* from either DE of S or internal ET in S, as evidenced by the fact that F_0 is proportional to [E_0] and intersects the F_0 axis at zero.

Another means of assessing the origin of the dansyl fluorescence observed in the stopped-flow experiments is to measure the equivalent of an excitation profile. This is obtained by plotting F_0/F_{bkg} from the stopped-flow experiments versus excitation wavelength. Such a profile will reveal which excitation wavelengths give rise to the dansyl fluorescence change. The results of such an experiment for the reaction of Dns-Gly-Tyr with carboxypeptidase A are shown in Figure 6. It can be seen that the only excitation maximum is found near 275 nm, which is at approximately the same wavelength as the excitation maximum of the enzyme (Figure 2A).

Effect of Products on LAP Catalysis. Before considering the stopped-flow experiments with LAP and its dansyl substrates, the effects of Leu and Gly-NHNH-Dns (the products of hydrolysis of Leu-Gly-NHNH-Dns) on LAP were examined. The rate of hydrolysis of Leu-*p*-nitroanilide (1 mM) by [(LAP)Zn_6M_6], where M = Zn(II), Mg(II), Mn(II), Cu(II), Ni(II), and empty, was measured in the presence of concentrations of each of these products that varied from 0–1 mM in 50 mM Tris and 0.1 M KCl, pH* 9, containing 5% methanol at 23 °C. The results (not shown) indicate that neither of these products inhibit any of the LAP species at any of these concentrations. The resutls for [(LAP)Zn_6Ni_6] and [(LAP)Zn_6Cu_6] were also not affected by the inclusion of 0.2 mM EDTA in the assays [see Lin et al. (1988b)]. Thus, no product inhibition or activation is observed for the reaction of LAP with Leu-Gly-NHNH-Dns.

Stopped-Flow Experiments with LAP. The optical and emission spectra of [(LAP)Zn_6M_6] and its dansyl substrates are very similar to those of carboxypeptidase A and Dns-Gly-Tyr, respectively. For this reason, these reactions were initially studied with the expectation that ET would be the dominant contribution to F_t when the system was excited near 280 nm (Van Wart & Lin, 1983). Before examining data for these reactions, however, it should be noted that Leu-Gly-NHNH-Dns differs from Dns-Gly-Tyr in that its absorption and emission spectra change appreciably on hydrolysis. The absorption of this substrate near 320 nm increases slightly on hydrolysis (not shown). The emission (λ_{ex} = 320 nm) and excitation (λ_{em} = 520 nm) spectra before and after hydrolysis (Figure 7) indicate that the fluorescence produced by this substrate on DE in the 280–330-nm region should decrease significantly on hydrolysis. Hence, a positive F_t that decreases on hydrolysis is expected due to the decay of [S], as given by the first term in eq 2, since $Q_S a_S$ is larger than $Q_P a_P$. However, since LAP and Leu-Gly-NHNH-Dns ostensibly have the same favorable properties for ET described above, changes in dansyl fluorescence due to the buildup and decay of ES_i are also possible.

The first experiments with LAP were carried out under conditions designed to maximize ET by employing excitation

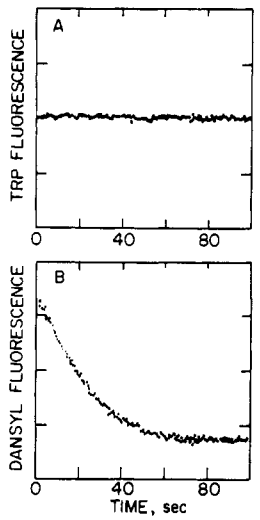

FIGURE 8: Stopped-flow fluorescence traces (λ_{ex} = 280 nm) showing the changes in (A) Trp and (B) dansyl fluorescence during the hydrolysis of 0.1 mM Leu-Gly-NHNH-Dns by 200 nM [(LAP)Zn_6Mg_6] in 50 mM Tris and 0.1 M KCl, pH 9 at 23 °C.

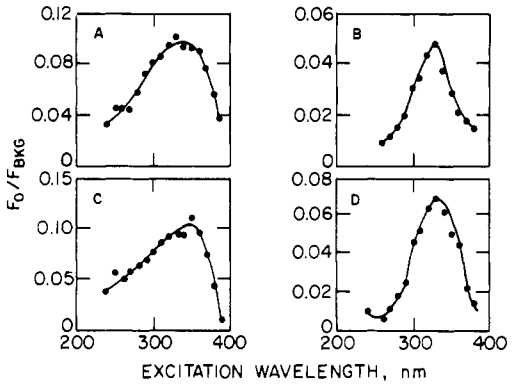

FIGURE 9: Excitation profiles for the maximum change in dansyl fluorescence (F_0) relative to the fluorescence background (F_{bkg}) for the hydrolysis of 0.1 mM Leu-Gly-NHNH-Dns by 0.2 μM [(LAP)Zn_6M_6] in 10 mM Tris and 0.1 M KCl, pH 9 at 23 °C, for M(II) = (A) Mg(II), (B) Mn(II), (C) Ni(II), and (D) Cu(II). The data for [(LAP)Zn_6Ni_6] and [(LAP)Zn_6Cu_6] were obtained in the presence of 0.2 mM EDTA.

at 280 nm. Stopped-flow traces showing the changes in both Trp and dansyl fluorescence on reaction of 0.1 mM Leu-Gly-NHNH-Dns with 0.2 μM [(LAP)Zn_6Mg_6] in 50 mM Tris and 0.1 M KCl, pH 9, at 23 °C [K_M = 0.33 mM (Lin et al., 1988b)] are shown in Figure 8. A significant decrease in dansyl fluorescence is observed on hydrolysis, but it is not accompanied by any change in Trp fluorescence, indicating that ET is unlikely to be responsible for the dansyl fluorescence change. The dependence of F_0 on [E_0] has been measured for this reaction by using excitation near the E absorption maximum at 280 nm and the S absorption maximum at 320 nm (data not shown). Within experimental error, F_0 is independent of [E_0] over a concentration range of hexamers of 0–1 μM at both excitation wavelengths, indicating that the changes in dansyl fluorescence are due to the first term in eq 2.

A series of stopped-flow fluorescence excitation profiles (λ_{em} = 520 nm) for the hydrolysis of Leu-Gly-NHNH-Dns by [(LAP)Zn_6M_6], where M = Mg(II), Mn(II), Cu(II), and Ni(II), are shown in Figure 9. All four profiles show that the maximal fluorescence changes are produced on DE of the dansyl group near 330 nm. These profiles may have a small shoulder in the 270–300-nm region that could be attributable to some ET from E. In contrast to the corresponding profile for carboxypeptidase A (Figure 6), however, the amount of

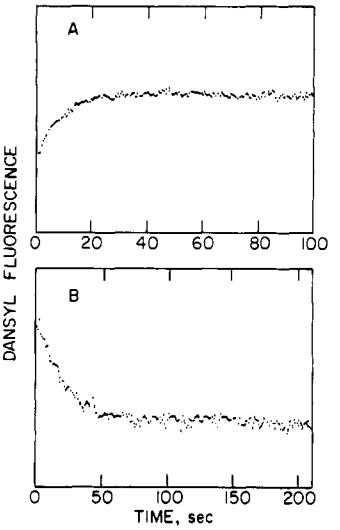

FIGURE 10: Stopped-flow fluorescence traces (λ_{ex} = 330 nm) for the hydrolysis of 200 μM (A) Leu-Gly-NH$(CH_2)_2$NH-Dns and (B) Leu-Gly-NH$(CH_2)_6$NH-Dns by 0.2 μM [(LAP)Zn_6Mg_6] in 50 mM Tris and 0.1 M KCl, pH 9 at 23 °C.

ET is very small. Subtraction of the excitation profiles for Leu-Gly-NHNH-Dns (S) and Gly-NHNH-Dns (P) shown in Figure 7B gives a spectrum very similar to those shown in Figure 9. Thus, the dansyl fluorescence changes in these reactions arise from the decay of S and are due to the differences in the absorption and emission properties of S and P upon their DE.

The same conclusion is reached for the hydrolysis of Leu-Gly-NH$(CH_2)_2$NH-Dns and Leu-Gly-NH$(CH_2)_6$NH-Dns by LAP. The emission (λ_{ex} = 330 nm) and excitation (λ_{em} = 560 nm) spectra of these two substrates recorded in 50 mM Tris and 0.1 M KCl, pH 9, before and after hydrolysis (not shown) are similar in shape to those shown for Leu-Gly-NHNH-Dns in Figure 7. However, the differences between $Q_S a_S$ and $Q_P a_P$ for excitation wavelengths in the 280–330-nm region are much smaller. Interestingly, the magnitude of the dansyl emission of Leu-Gly-NH$(CH_2)_2$NH-Dns increases slightly on hydrolysis, while that of Leu-Gly-NH$(CH_2)_6$NH-Dns decreases slightly for all excitation wavelengths in the 280–330-nm region. Accordingly, the stopped-flow fluorescence traces for the hydrolysis of these two substrates by [(LAP)Zn_6Mg_6] obtained with 330-nm excitation show these same trends (Figure 10). Similar traces with lower F_0 values are obtained with excitation near 280 nm, but there is no accompanying change in the fluorescence of LAP near 330 nm. The F_0 values are also independent of [E_0] at both excitation wavelengths. The *increase* in dansyl fluorescence observed on hydrolysis of Leu-Gly-NH$(CH_2)_2$NH-Dns cannot be explained by the ET mechanism. In summary, the stopped-flow fluorescence changes observed on hydrolysis of all three dansyl substrates by LAP are the consequence of DE of S and P and reflect changes in the concentrations of S rather than ES_i.

Possible Perturbations of the Energy Relay System with LAP and Its Dansyl Substrates. The failure to observe any appreciable ET in these experiments with LAP is somewhat surprising, particularly since Auld and Prescott (1983) have reported stopped-flow ET behavior for *Aeromonas* aminopeptidase with the similar substrate Leu-Ala-NH$(CH_2)_2$NH-Dns. This suggests that something is disturbing the energy relay system in the ES_i for the reactions studied here. One possibility is that the fluorescence of LAP is quenched by the excess M(II) used to prepare the [(LAP)Zn_6M_6], as demonstrated elsewhere for Cu(II) and Ni(II) (Lin & Van Wart,

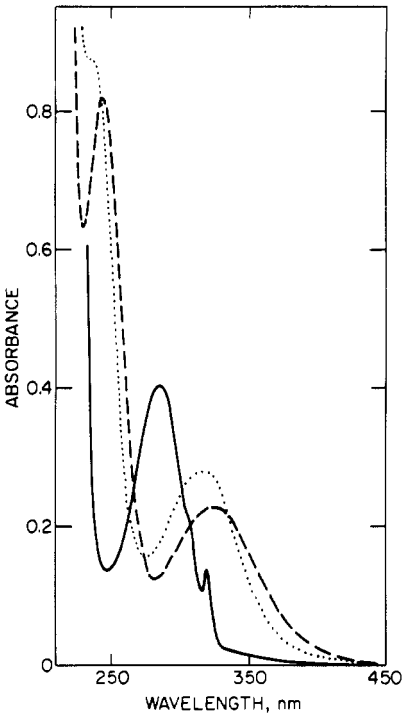

FIGURE 11: Absorption spectra of the FH^{3+} (—), FH^{2+} (---), and FH^{+} (···) forms of Leu-Gly-NHNH-Dns in 0.1 M phosphate at pH 1, 6, and 9, respectively, at 23 °C.

1988). This cannot be the case here, however, because Mg(II), Mn(II), and Zn(II) are not effective quenchers and EDTA is included in the buffer in all reactions with Cu(II) and Ni(II) to chelate all excess M(II) [see details in Lin et al. (1988b)].

An alternative possibility is that the spectral properties of the dansyl group of S in the ES_i are altered so that the absorbance near 330 nm is shifted or the fluorescence near 520–560 nm is abolished. This could be due to a change in the environment of S on binding to LAP. For example, protonation or deprotonation of fluorescent substrates containing ionizable groups may cause dramatic changes in their optical and emission spectra. When bound to the E, the state of protonation of such S at any pH can be greatly influenced by interactions with nearby acidic or basic amino acid residues near the active site. Therefore, the spectra of S in ES_i can be quite different from those of free S.

For example, Chen and Kernohan (1967) have observed dramatic changes in the emission spectrum and quantum yield for dansylamine when bound to bovine carbonic anhydrase B. Dansylamine is an inhibitor that forms a stable 1:1 complex with the enzyme. In water, dansylamine has an emission maximum at 580 nm and a quantum yield of 0.055. On binding to the enzyme, there is a blue shift in the fluorescence maximum to 468 nm and a large increase in quantum yield to 0.84. These changes in emission properties on formation of this enzyme–inhibitor complex have been attributed to the loss of a proton on complexation. Similar changes in the emission properties of free dansylamine are observed when it is deprotonated by raising the pH. Thus, the investigation of the dependence of the spectra of S on pH can foretell possible changes in its properties on binding to the E.

Dramatic changes in the absorption and fluorescence spectra of Leu-Gly-NHNH-Dns are observed on variation of the pH (Figure 11). The absorption band near 320 nm at pH 9 shown in Figure 3 is first red-shifted to 328 nm and decreased in intensity as the pH of the solution is decreased. Next, as the pH drops below 5, a completely different absorption spectrum starts to appear. This spectrum is more structured than that

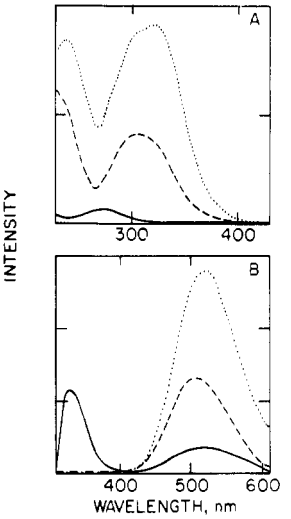

FIGURE 12: (A) Emission spectra (λ_{ex} = 280 nm) and (B) excitation spectra (λ_{em} = 520 nm) for the FH^{3+} (—), FH^{2+} (---), and FH^{+} (···) forms of Leu-Gly-NHNH-Dns in 0.1 M phosphate at pH 1, 6, and 9, respectively, at 23 °C.

of the higher pH forms. A new band appears at 287 nm with a shoulder at 306 nm and a sharp peak at 320 nm. A plot of the absorbance at 285 nm as a function of pH shows that there are three pH-dependent species that interconvert, with pK_a values of approximately 3.9 and 7.2. The absorption spectra of these three forms of Leu-Gly-NHNH-Dns found at pH 1 (FH^{3+}), 6 (FH^{2+}), and 9 (FH^{+}) are shown in Figure 11.

The emission (λ_{ex} = 280 nm) and excitation (λ_{em} = 520 nm) spectra of Leu-Gly-NHNH-Dns also change markedly with pH in the 1–10 range (Figure 12). The intensity of the emission band near 520 nm at pH 9 shown in Figure 3 decreases markedly as the pH of the solution is decreased and shifts to 510 nm at pH 6. A new emission band centered at 335 nm starts to appear at pH 5 and increases in intensity as the pH is lowered to 1. A titration curve of the fluorescence intensity at 510 nm clearly shows that two protonic equilibria are involved, with pK_a values of 4.4 and 7.3. The excitation spectra also undergo pH-induced changes with these same values of pK_a. This indicates that at least three different forms of the substrate with different degrees of protonation exist in equilibrium over the pH 1–10 range. The emission spectra (λ_{ex} = 280 nm) and excitation spectra (λ_{em} = 520 nm) of these species at pH 1 (FH^{3+}), 6 (FH^{2+}), and 9 (FH^{+}) are shown in Figure 12.

Other dansyl compounds, including Leu-Gly-NH-$(CH_2)_2$NH-Dns and Leu-Gly-NH$(CH_2)_6$NH-Dns, show similar changes in their emission spectra as a function of pH. Unlike Leu-Gly-NHNH-Dns, however, only one protonic equilibrium with a pK_a value in the 3.6–4.5 range is found for these compounds. A summary of the pK_a values for these compounds and their absorption and emission spectra at pH 1 is given in Table I. The similarity in the spectral properties of all of these dansyl species at low pH suggests that the pK_a value near 4 corresponds to the dimethylamino group on the dansyl moiety, which is similar to the pK_a value of 3.69 for 5-aminonaphthalene-1-sulfonic acid (Brown et al., 1955). Since only Leu-Gly-NHNH-Dns exhibits the pK_a near 7.4, this is attributed to the hydrazide group. In support of this assigment is the observation that diethylhydrazine has a pK_a of 7.78 (Hinman, 1958).

Leu-Gly-NHNH-Dns has three groups and Leu-Gly-NH-$(CH_2)_2$NH-Dns and Leu-Gly-NH$(CH_2)_6$NH-Dns each have two groups that are expected to undergo ionization in the pH

Table I: Summary of pK_a Values Obtained from Fluorescence Titrations[a] and Spectral Properties at pH 1 of Several Dansyl Species

dansyl species	pK_a^1	pK_a^2	pH 1 $(\lambda_{em})_{max}$[b]	pH 1 $(\lambda_{abs})_{max}$
Leu-Gly-NHNH-Dns	4.4	7.3	335 (72),510 (21)	287,320
Leu-Gly-NH-$(CH_2)_2$NH-Dns	3.8		336 (80),560 (52)	286,320
Leu-Gly-NH-$(CH_2)_6$NH-Dns	3.6		336 (80),560 (48)	286,320
Dns-Ala	4.0		335 (52),568 (17)	287,321
Dns-OH	4.5		333 (82),510 (23)	273,283,319

[a] All spectra were excited at 280 nm. [b] The numbers in parentheses represent the relative intensities of the bands in the spectrum of each compound relative to each other.

range of 1–10. All three S have α-amino groups that normally have pK_a values near 9.5 and the dansyl dimethylamino group which has a characteristic pK_a near 4. In addition, Leu-Gly-NHNH-Dns has a hydrazide bond that is expected to have a pK_a value near neutrality. Apparently, the ionization of the α-amino groups of these three substrates does not influence their fluorescence spectra, and the changes observed are due to the other ionizations. Thus, for Leu-Gly-NH$(CH_2)_2$NH-Dns and Leu-Gly-NH$(CH_2)_6$NH-Dns, the equilibrium that influences the emission spectra is

$$FH^{2+} \overset{pK_a}{\rightleftharpoons} FH^{+} \qquad (6)$$

where the pK_a value of 4 corresponds to that of the dansyl group. For Leu-Gly-NHNH-Dns, on the other hand, the equilibria that affect the fluorescence are

$$FH^{3+} \overset{pK_a^1}{\rightleftharpoons} FH^{2+} \overset{pK_a^2}{\rightleftharpoons} FH^{+} \qquad (7)$$

where pK_a^1 and pK_a^2 correspond to the dansyl and hydrazide groups, respectively.

It is noteworthy that the FH^{3+} form of Leu-Gly-NHNH-Dns and FH^{2+} forms of Leu-Gly-NH$(CH_2)_2$NH-Dns and Leu-Gly-NH$(CH_2)_6$NH-Dns absorb maximally at 280 nm and have emission maxima near 335 nm. These parameters are quite different from those of the FH^{+} form of these S at pH 9 but are very similar to those of LAP. This near coincidence of the absorption and emission spectra of this form of S with those of E would prevent ET if S were converted to this "protonated" form at pH 9 through interaction with amino acid residues of E in the ES_i. This is one possible explanation for the lack of ET in the stopped-flow fluorescence experiments of the hydrolysis of these dansyl substrates by LAP.

The experimental observations delineated above clearly indicate that E-to-S ET can, at best, contribute a very minor amount to the fluorescence changes observed during the reaction of $[(LAP)Zn_6M_6]$ with the three dansyl substrates studied here. This surprising result underscores the need to establish the origin of fluorescence changes in such experiments before data analysis, even though the E–S system in question may have properties that closely mimic those of similar systems that do exhibit ET. The great majority of the fluorescence changes observed on hydrolysis of these substrates by LAP, particularly on excitation in the 330-nm region, are due to the differences in the optical properties of S and P. Thus, to study the steady-state hydrolysis of these dansyl substrates by $[(LAP)Zn_6M_6]$, DE within the dansyl excitation band near 330 nm should be employed for maximum sensitivity. Since the largest changes are observed for Leu-Gly-NHNH-Dns, most of the steady-state kinetic analysis will be carried out with this S and the resulting data analyzed by kinetic equations derived in the following paper (Lin et al., 1988b).

Registry No. LAP, 9001-61-0; Leu-Gly-NHNH-Dns, 89315-19-5; Leu-Gly-NH$(CH_2)_2$NH-Dns, 114333-74-3; Leu-Gly-NH-$(CH_2)_6$NH-Dns, 114333-75-4; Dns-Ala, 35021-10-4; Dns-OH, 4272-77-9; Dns-Gly-Tyr, 31944-24-8; carboxypeptidase A, 11075-17-5.

REFERENCES

Auld, D. S. (1977) *Bioorganic Chemistry* (Van Tamelin, E. E., Ed) Vol. 1, pp 1–17, Academic, New York.

Auld, D. S., & Holmquist, B. (1974) *Biochemistry 13*, 4355–4361.

Auld, D. S., & Prescott, J. M. (1983) *Biochem. Biophys. Res. Commun. 111*, 946–951.

Auld, D. S., Latt, S. A., & Vallee, B. L. (1972) *Biochemistry 11*, 4994–4999.

Brown H. C., McDaniel, D. H., & Hafliger, O. (1955) *Determination of Organic Structure by Physical Methods* (Braude, E. A., & Nachod, F. C., Eds.) p 599, Academic, New York.

Chance, B. (1974) *Techniques of Chemistry* (Hammes, G. G., Ed.) Vol. 6, pp 5–62, Wiley-Interscience, New York.

Chen, R. F., & Kernohan, J. C. (1967) *J. Biol. Chem. 242*, 5813–5823.

Delange, R. J., & Smith, E. L. (1971) *Enzymes (3rd Ed.) 3*, 81–118.

Galdes, A., Auld, D. S., & Vallee, B. L. (1983) *Biochemistry 22*, 1888–1893.

Galdes, A., Auld, D. S., & Vallee, B. L. (1986) *Biochemistry 25*, 646–651.

Gibson, Q. H. (1969) *Methods Enzymol. 16*, 187–228.

Himmelhoch, S. R. (1970) *Methods Enzymol. 19*, 508–513.

Hinman, R. L. (1958) *J. Org. Chem. 23*, 1587–1588.

Latt, S. A., Auld, D. S., & Vallee, B. L. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 1383–1389.

Lin, W.-Y., & Van Wart, H. E. (1988) *J. Inorg. Biochem. 32*, 21–38.

Lin, W.-Y., Lin, S. H., Morris, R. J., & Van Wart, H. E. (1988a) *Biochemistry* (third of three papers in this issue).

Lin, W.-Y., Lin, S. H., & Van Wart, H. E. (1988b) *Biochemistry* (second of three papers in this issue).

Lobb, R. R., & Auld, D. S. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2684–2688.

Lobb, R. R., & Auld, D. S. (1980) *Biochemistry 19*, 5297–5302.

Lobb, R. R., & Auld, D. S. (1984) *Experientia 40*, 1197–1206.

Mattis, J. A., & Fruton, J. S. (1976) *Biochemistry 15*, 2191–2194.

Morgan, G., & Fruton, J. S. (1978) *Biochemistry 17*, 3562–3568.

Sachdev, G. P., & Fruton, J. S. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3424–3427.

Schechter, I., & Berger, A. (1967) *Biochem. Biophys. Res. Commun. 27*, 156–162.

Van Wart, H. E., & Lin, S. H. (1981) *Biochemistry 20*, 5682–5689.

Van Wart, H. E., & Zimmer, J. (1981) *Anal. Biochem. 117*, 410–418.

Van Wart, H. E., & Lin, S. H. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 7506–7509.

Williams, A. C., & Auld, D. S. (1986) *Biochemistry 25*, 94–100.