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Interaction of α-Dansylated Peptide Inhibitors with Porcine Pepsin: Detection of Complex Formation by Fluorescence Energy Transfer and Chromatography and Evidence for a Two-Step Binding Scheme†

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ABSTRACT: Peptide inhibitors, specifically labeled at the α-amino terminus by dansylation, have been prepared by utilizing solid-phase peptide synthesis. Changes in fluorescence have been observed upon mixing these peptides with porcine pepsin that can be attributed to the formation of at least two complexes. Energy transfer between tryptophan residues of the protein and the dansyl group of the inhibitors has been detected by the unique excitation spectra generated. The kinetics of formation of the second complex can be correlated with inhibition of the catalytic activity of pepsin. Evidence for complex formation has also been obtained from gel filtration experiments using the fluorescent peptides.

Fragments of the amino-terminal region of pepsinogen are released upon conversion to pepsin. Amino acids from residues 1 to 44 of porcine pepsinogen are released as peptides of varying length depending on the conditions (Dunn et al., 1978). The first fragment released is peptide (1-16) as shown by trapping studies with the use of a specific inhibitor (Dykes & Kay, 1976).

Peptide (1-16), as well as several other fragments, will inhibit the pepsin-catalyzed clotting of a dilute solution of milk (Herriott, 1938, 1939, 1941; Dunn et al., 1978). Inhibition of the cleavage of small, synthetic peptides can also be observed.[1] The molecular details of this inhibition process are unclear at this point. Since preliminary kinetic studies have indicated a complex pathway leading to inhibition, we have sought further information on this process by the use of the fluorescent reporter group method (Stryer, 1978). We have prepared a series of derivatives of the peptide (1-16) sequence

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[1] B. M. Dunn and C. Deyrup, unpublished observation.

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by solid-phase peptide synthesis to examine the role of various amino acids in the inhibition (Dunn & Deyrup, 1979). This method presents us with an opportunity to selectively add the (dimethylamino)naphthalenesulfonyl (dansyl or Dns) at the amino terminus of these peptide chains, since all side chains are protected in this procedure. We have prepared α-dansylated peptides and examined their interaction with porcine pepsin by fluorescence methods. Our choice of the α-amino group as the point of addition of the dansyl group was also dictated by the observation of Kassell and co-workers (Harish-Kumar & Kassell, 1977), who demonstrated that the amino-terminal leucine of peptide (1–16) could be removed by the Edman degradation without any effect on the inhibitory potency of the peptide. Therefore, a substitution of that locus should be without effect on the activity. This has been confirmed by these studies.

Materials and Methods

Synthesis. All peptides were prepared by the solid-phase methods with specific alterations in the standard procedure (Barany & Merrifield, 1979) indicated below. The dansylation procedure is described for peptide (1–16) and is the same for the other peptides.

α-Dns-(1–16). After removal of the *t*-Boc protecting group from the α-amino of Leu at position 1 of the protected peptide bound to Merrifield resin with 25% trifluoroacetic acid (F_3-AcOH) in CH_2Cl_2 and washing with CH_2Cl_2, the amino group was neutralized with 10% triethylamine (TEA) in CH_2Cl_2. A 10-fold excess of dansyl chloride was dissolved in CH_2Cl_2–TEA (2:1) and added to the peptidylresin. This was stirred or shaken for 30 min and then washed with CH_2Cl_2 and methanol. The resulting resin was dried by suction for 1 h then placed in a vacuum desiccator overnight. The dansylated peptide was cleaved from the polystyrene resin, and all side chain protecting groups were removed by treatment with anhydrous HF in a ToHo KaSEI apparatus at 0 °C for 1 h. Ten milliliters of liquid HF was used per g of peptidyl resin, and 1 mL of anisole per gram resin was added as a cation scavenger. The HF was removed with a water aspirator and a N_2 stream. Anisole was extracted by several ethyl acetate washes. The crude peptide was then extracted with 50% acetic acid–H_2O. The acetic acid was removed by rotary evaporation and the sample was lyophilized.

Purification of Dansylated Peptides. In all cases the dried peptide sample was redissolved in a 1 M acetic acid solution and passed through a Sephadex G-10 or G-15 column to remove small peptides or free amino acids. The major peak of peptide was pooled and lyophilized. The sample was then dissolved in 0.01 M ammonium acetate at pH 5.0 and applied to a 0.9 × 40 cm column of CM-Sepharose equilibrated with the same buffer. A gradient passing from 0.01 to 1.0 M is used to elute the basic peptides. Peaks were pooled and examined by amino acid composition and thin-layer chromatography.

Final purification for α-Dns-(1–12) was provided by HPLC using a Waters high-performance liquid chromatograph consisting of two Model 6000A solvent delivery systems, a Model 660 solvent programmer, a Model 440 absorbance detector, and a Model 450 variable-wavelength absorbance detector, a U6K injector, and a μBondapak C_{18} reverse-phase column. Solvent A was 10% 2-propanol, 12.5% acetic acid, and 77.5% H_2O. Solvent B was 31.5% 2-propanol, 12.5% acetic acid, and 56% H_2O. Immediately after injection of up to 150 μL of peptide in solvent A, a 20-min concave gradient was run from 10% to 80% solvent B at 1 mL/min, yielding a peak at 12 min followed by a larger peak at 15 min. The detectors were at 280 and 254 nm. Amino acid analysis of the first peak is low in Leu and Ser while the analysis of the second peak was consistent with the desired sequence (Table I). Purity was also checked by thin-layer chromatography (TLC) on cellulose plates with a solvent of butanol–acetic acid–water (4:1:5). A single spot with an R_f of 0.6 was seen by fluorescence and ninhydrin detection.

Table I: Amino Acid Analyses of Peptides Used in This Study[a]

	peptide		
	α-Dns-[Thr^8]-(1–16)	α-Dns-(1–12)	α-Dns-[Gly^5]-(1–12)
Lys	3.34 (3)	3.05 (3)	2.75 (3)
Arg	1.11 (1)	0.95 (1)	0.95 (1)
Asp	1.07 (1)	– (0)	– (0)
Thr	0.88 (1)	– (0)	– (0)
Ser	0.85 (1)	0.79 (1)	0.87 (1)
Glu	1.01 (1)	– (0)	– (0)
Pro	present[b] (1)	present[b] (1)	– (0)
Gly	– (0)	– (0)	1.25 (1)
Val	2.92 (3)	3.18 (3)	3.10 (3)
Leu	3.83 (3–4)[c]	2.03 (2–3)[c]	2.08 (2–3)[c]

[a] A portion of a pooled sample was lyophilized, and 1 mL of 6 M HCl was added with 1 μL of mercaptoethanol and 10 μL of 1% phenol. The samples were then evacuated on a vacuum line and sealed with a torch. They were heated at 116 °C for 24–48 h, the HCl was evaporated with a vacuum line equipped with a dry ice trap and NaOH trap, and the residue was redissolved in a 0.2 N sodium citrate solution (pH 2.2 with thiodiglycol and pentachlorophenol). The expected ratios are given in parentheses. A dash indicates no peak was observed. [b] Peak observed with correct time but not integrated. [c] The amount of leucine is variable in our hydrolyses, apparently because there may be some hydrolysis of the dansyl-Leu bond depending on the length of hydrolysis.

A pooled peak from the CM-Sepharose column of α-Dns-[Gly^5]-(1–12) with the best amino acid composition was lyophilized and then dissolved in butanol–acetic acid–water (4:1:5). This sample was then passed through a column of microcrystalline cellulose packed in the same solvent. A conservative cut from the resulting broad peak gave a single spot in the TLC system described above and gave a single major peak in HPLC using the system described above. The analysis is given in Table I along with the analyses of the other peptides. Peptide α-Dns-(1–16) after CM-Sepharose chromatography gave peaks at the correct times on the amino acid analyzer for Lys, Arg, Asp, Ser, Glu, Pro, Val, and Leu but these were not integrated. This sample was used for preliminary experiments reported here. The major observations made with this peptide were reproduced with the other peptides prepared later.

Fluorescence Experiments. Excitation and emission spectra were recorded on an Aminco-Bowman spectrophotofluorometer at 25 °C. Concentrations of α-dansyl peptides were chosen to yield absorbances of less than 0.07 at 335 nm and less than 0.04 at 290 nm. Slits of 1 nm were chosen for these experiments. Extinction coefficients were determined by using a Cary 15 spectrophotometer.

Activity Assays. The catalytic assay of free pepsin was carried out by using the milk clotting assay (Dunn et al., 1978). Inhibition assays were carried out by mixing a peptide sample with a standard amount of pepsin, incubating at 37 °C for 10 min, and then assaying an aliquot for residual pepsin activity. Kinetic experiments established that these conditions lead to equilibrium binding. Assays of pepsin mixed with dansyl peptides for fluorescence experiments were carried out by taking an aliquot from the fluorescence cuvette and measuring the pepsin activity present with *no* incubation at 37 °C.

Chromatography. The interaction of some peptides with pepsin was studied by using a 0.9 × 40 cm Sephadex G-50

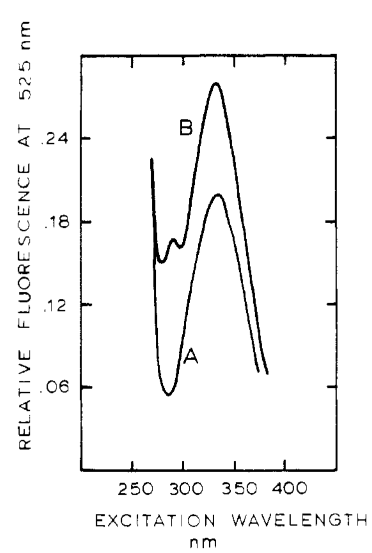

FIGURE 1: Fluorescence excitation curves for α-Dns-(1–16) obtained by measuring fluorescence at 525 nm. Curve A is the peptide alone (3.52×10^{-5} M) at pH 5.5 and curve B is the spectrum when 0.5 equiv of pepsin was added.

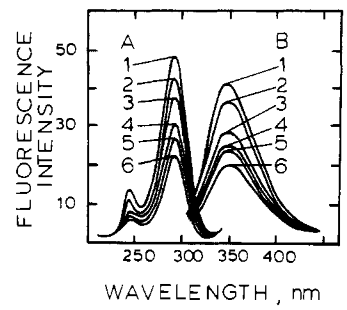

FIGURE 2: Fluorescence excitation curves (A) and emission curves (B) for porcine pepsin (at 1.1×10^{-6} M). The excitation curves were obtained by measuring fluorescence at 340 nm while scanning the excitation wavelength from 200 to 330 nm. The emission curves were obtained by setting the excitation wavelength at 290 nm and scanning the resulting fluorescence from 300 to 450 nm. The curves labeled 1 are from free pepsin. The curves labeled 2, 3, 4, 5, and 6 are obtained after the addition of 0.63, 1.9, 2.53, 3.48, and 5.69 equiv of peptide α-Dns-(1–16).

column in 0.1 M sodium acetate. Samples of pepsin and peptide were incubated for 10 min at 37 °C and then applied to the column and eluted with the same buffer. Pepsin was detected by absorbance at 280 nm and by the activity assay while the dansyl peptide was detected by emission at 525 nm with excitation at 340 nm.

Results

All peptides reported in this study inhibited pepsin. The apparent K_I values were 0.3 μM for α-Dns-(1–16) and 0.45 μM for α-Dns-(1–12) based on plots of inhibition vs. concentration of peptide. Since the value of the apparent K_I for peptide (1–16) is 0.25 μM (Dunn et al., 1978), the dansyl group has little effect upon the inhibition. Replacement of Pro^5 by Gly in α-Dns-[Gly^5]-(1–12) resulted in a reduction in binding affinity, and the K_I value was determined to be 5.0 μM (Dunn & Deyrup, 1979). This has been confirmed by comparison of the undansylated (1–12) peptides with Gly in positions 5, 4 and 5, 6 and 7, and 4–7 (Dunn & Lewitt, 1980).

All dansyl peptides used in this study exhibit similar fluorescence properties at pH 5.5 in sodium acetate buffer. The excitation maximum has been observed to occur between 335 and 340 nm, and the emission maximum peaks at 525–535 nm. These values are consistent with other reports of α-dansylated peptides (Stryer & Haugland, 1967).

When pepsin is added to these peptides, a striking change is observed in the excitation spectra. An additional excitation peak is observed in the 290-nm region. As shown in Figure 1 for α-Dns-(1–16), since the free dansyl peptide exhibits a deep trough at that point in the excitation spectrum, this new peak must arise from absorption by pepsin. Since the emission at 525 is being measured, this new excitation maximum must derive from fluorescence energy transfer from the protein chromophores to the peptide dansyl group. Pepsin has an emission maximum at 340 nm and no emission above 500 nm.

If energy is being lost from Trp of pepsin to dansyl of the peptides, then less energy can show up as Trp fluorescence. This was confirmed by the reciprocal experiment shown in Figure 2 where pepsin is titrated with small amounts of α-Dns-(1–16) and the intense pepsin fluorescence is seen to be quenched. Both excitation and emission curves are shown and both are quenched. The reduction in fluorescence is at least 10 times greater than dilution effects.

Several control experiments have been done to confirm the energy transfer assignment. First, dansylleucine has similar fluorescence properties to the dansyl peptides described here. When dansylleucine was exposed to addition of pepsin under the identical conditions of concentration and total fluorescence intensity, no energy transfer band was observed. Second, α-Dns-(1–16) has been titrated with bovine serum albumin. This protein is also quite acidic and has multiple Trp residues. Amounts of BSA equivalent to the amounts of pepsin used did not generate any new excitation band at 290 nm. Thus the energy transfer band appears to be due to the specific interaction between pepsin and the dansyl peptides. Since these experiments were conducted with concentrations of peptides and proteins below 100 μM, it is unlikely that trivial emission and reabsorption are occurring.

The observation of a new excitation maximum was first obtained by preparing equilibrium mixtures of pepsin and peptide. Previous experiments have established that incubation of pepsin and an inhibitory peptide at 37 °C for 10 min was sufficient to reach equilibrium. In later experiments, however, we mixed pepsin with the dansyl peptide at 25 °C and began measuring excitation curves with observation at 525 nm as rapidly as possible. This lead to the finding that, at early times after mixing, an even more intense energy transfer band was seen for α-Dns-[Thr^8]-(1–16) plus pepsin. This has also been observed for α-Dns-(1–12) and α-Dns-[Gly^5]-(1–12).

When the mixtures were scanned at various times from mixing to 60 min, the excitation curves decrease until a spectrum is reached when the excitation curve is identical with the curves obtained after incubation at 37 °C for 10 min. The changes describe a classic A → B → C pattern, where A is free α-dansyl peptide, B is the first species formed within several seconds of mixing, and C is the final, equilibrium state, formed from B in a process requiring a time scale of minutes.

Since the time scale for this second fluorescence step was similar to the time scale we have observed for inhibition kinetics,[1] we have studied the course of inhibition of pepsin by these peptides while the fluorescence spectra were being run. After α-Dns-[Thr^8]-(1–16) peptide and pepsin were mixed, spectra were taken at 5-min intervals and aliquots were assayed for pepsin activity. The resulting activities are converted to percent inhibition by comparison with an identical sample of pepsin without inhibitor which was equivalent to the activity seen immediately after mixing. The fluorescence intensity and percent inhibition are plotted in Figure 3 (upper panel) for α-Dns-[Thr^8]-(1–16) plus pepsin and in Figure 3 (lower panel) for α-Dns-(1–12) plus pepsin. In both cases, the slow, second

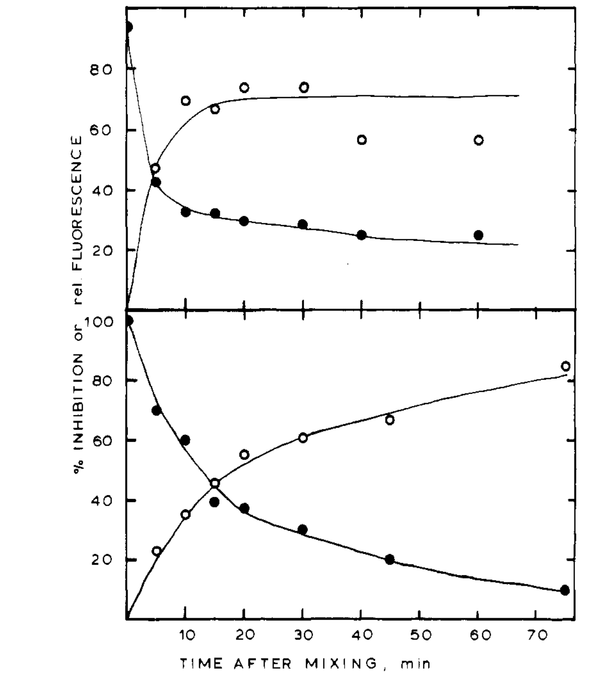

FIGURE 3: (Upper panel) Plot of relative fluorescence intensity at 525 nm with excitation at 290 nm for peptide α-Dns-[Thr[8]]-(1–16) plus pepsin vs. time (●). Also plotted is the percent inhibition of the pepsin vs. time (○). Peptide is 5.6×10^{-5} M and pepsin is 1.12×10^{-5} M. (Lower panel) Plot of the relative fluorescence intensity at 525 nm with excitation at 290 nm for peptide α-Dns-(1–12) plus pepsin vs. time (●). Also plotted is the percent inhibition of the pepsin vs. time (○). Peptide is 1.55×10^{-5} M and pepsin is 3.1×10^{-6} M.

step involving a decrease in fluorescence intensity (emission at 525 nm; excitation at 290 nm) correlated well with the appearance of inhibition.

The change in fluorescence at 525 nm after mixing pepsin with α-Dns-[Thr[8]]-(1–16) was also followed by observing the time course with fixed excitation at 290 nm. The resulting curves (not shown) show a very rapid increase, within the manual mixing time of 3–5 s, followed by a slow decrease. The decreasing curves could be fit to a first-order kinetic expression. This suggests that the second, slow step is a unimolecular reaction of the complex of pepsin and peptide.

Calculation of Distances between Chromophores Utilizing Energy Transfer Data. The distance between interacting chromophores is given by

$$r = (E^{-1} - 1)^{1/6} R_0$$

where E is the efficiency of transfer and R_0 is a distance where the transfer will be 50% efficient (Stryer, 1978). R_0 is equal to 21 Å for the Trp–Dns pair (Stryer, 1959; Steinberg, 1971). Since Trp shows low polarization, the use of an orientation factor of $^2/_3$ is justified (Haas et al., 1978). The term E is given by

$$E = \frac{\epsilon_A C_A}{\epsilon_D C_D}\left(\frac{F_{D+A}}{F_A} - 1\right)$$

where ϵ_A and C_A are the extinction coefficient at 290 nm and concentration of the dansyl peptide, ϵ_D and C_D are the extinction coefficient at 290 nm and concentration of the protein, F_A is the intensity of fluorescence at 525 nm with excitation at 290 nm of the dansyl peptide, and F_{D+A} is the intensity of fluorescence at 525 nm with excitation at 290 nm of the combination of pepsin and dansyl peptide. To obtain the fluorescence of the 1:1 complex of peptide and protein, it was necessary to obtain data at several ratios of peptide to pepsin. For α-Dns-[Thr[8]]-(1–16) a plot of the reciprocal of the

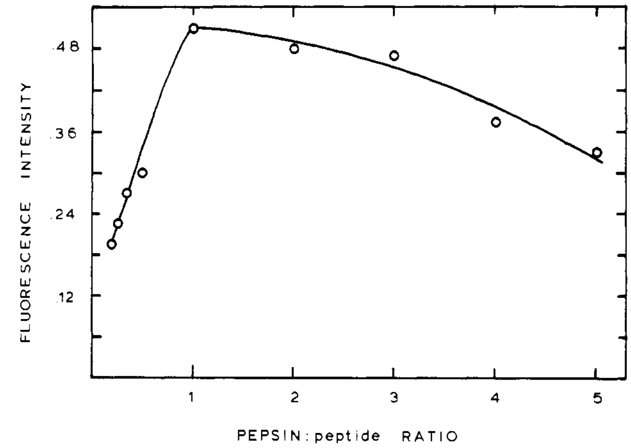

FIGURE 4: Plot of the fluorescence intensity at 525 nm with excitation at 290 nm for peptide α-Dns-(1–12) at 1.32×10^{-5} M and pepsin vs. the molar ratio of pepsin to peptide.

fluorescence intensity vs. the reciprocal of the protein concentration gave a straight line with a y intercept equal to the reciprocal of the maximal fluorescence. This procedure was employed for both the initial large fluorescence and the equilibrium fluorescence for this peptide to obtain the maximal values. For the (1–12) peptides, higher concentrations of peptide were employed. When the fluorescence change was observed at various ratios of pepsin to peptide α-Dns-(1–12), the value reached a plateau at a 1:1 ratio (Figure 4). This value was used in the calculation. At very high pepsin:peptide ratios (3 or higher) the fluorescence drops off, probably due to inner filter effects by the protein. The resulting distances are 18 Å for the initial species B and 27 Å for the equilibrium species C for α-Dns-[Thr[8]]-(1–16) and 28 Å for the initial species B and 36 Å for the equilibrium species C for α-Dns-(1–12). The magnitude of these number is only accurate to ±20% due to the inherent errors in the energy transfer procedure (Dale et al., 1979). Of greatest significance is the apparent change of 8–9 Å in each case between the initial complex and final complex.

Gel Filtration Chromatography of Mixtures of Dansyl Peptides and Pepsin. A mixture of pepsin and dansylated peptide was incubated at 37 °C for 10 min and applied to a column of Sephadex G-50 that had been shown to separate the protein (M_r 35000 eluting at 13–14 mL) from free peptide (M_r 2000 eluting at 26 mL). All fractions were scanned for fluorescence emission at 525 nm with excitation at 340 nm. The results of such an experiment are shown in the upper panel of Figure 5 for α-Dns-(1–12). It can be seen that considerable dansyl fluorescence comigrates with the protein peak. This provides independent evidence for complex formation of sufficient stability to survive a molecular sieve separation. A second experiment was performed in which the tertiary mixture of pepsin, α-Dns-(1–12), and pepstatin, a bacteria peptide known to bind to the active site of acid proteases, was chromatographed. The resulting column is shown in the lower half of Figure 5. It may be seen that amount of dansyl fluorescence and, hence, the amount of bound peptide are greatly reduced by the presence of pepstatin. A similar result was obtained when pepsin was first incubated with pepstatin followed by addition of α-Dns-(1–12) or when the enzyme was first incubated with α-Dns-(1–12) followed by pepstatin. The enzyme activity measured with 10-μL aliquots of the fractions is approximately 5% of the activity that would be observed for that amount of pepsin in the absence of the inhibitors.

A similar result was obtained for peptide (1–16) by measuring the reduction in activity of the pepsin peak when

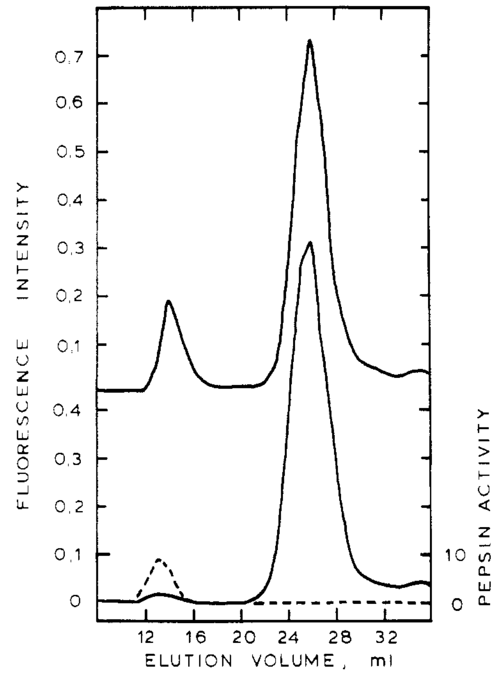

FIGURE 5: Plot of the fluorescence at 525 nm with excitation at 340 nm vs. elution volume for the mixture of α-Dns-(1–12) and pepsin (upper curve) and the mixture of α-Dns-(1–12), pepsin, and pepstain (lower curve). The dansyl peptide (0.144 μmol) was present in a 4-fold excess over pepsin in each experiment. The milk clotting activity of a 10-μL aliquot of each fraction is also plotted in the lower panel (---).

cochromatographed with that peptide.

Discussion

Addition of the dansyl chromophore to the amino terminus of the peptides related to the 1–16 segment of pepsinogen has not affected the capacity of these peptides to inhibit pepsin. This is consistent with Kassell's report (Harish-Kumar & Kassell, 1977) that Leu[1] is not essential to the inhibitory function. It is also in accord with our observations that the critical sequence of these inhibitory peptides is the 4–7 region (Dunn & Lewitt, 1980).

The changes in fluorescence reported for these peptides when mixed with pepsin are consistent with the formation of complexes. This provides compelling physical evidence that the inhibitory effect of these peptides on the milk clotting action of pepsin is due to a physical interaction with the enzyme rather than a perturbation of the substrate. We have also observed similar inhibition of the hydrolysis of small defined peptide substrates.[1]

The two-phase change in fluorescence intensity upon interaction with pepsin deserves further comment. First, this is completely consistent with our observation of a complex kinetic pathway for inhibition (Dunn & Deyrup, 1979). The rate of loss of pepsin activity shows a biphasic dependence on the concentration of the inhibiting peptide. Rich and his colleagues (Rich & Sun, 1977) have also observed complex kinetics for the reaction of pepstatin with pepsin. Second, this biphasic process could have several origins. The fluorescence results reported here imply that an initial complex is formed with greatly elevated fluorescence and undergoes slow conversion to a more stable complex with intermediate fluorescence. The nature of this slow conversion remains obscure. Since the appearance of inhibition correlates quite well with the second, slow step of the kinetics, the slow fluorescence step is of major significance in understanding the inhibition process. The peptide could bind in several ways to the protein to form the initial complex. It could bind nonspecifically to the surface through electrostatic attraction. This might juxtapose the dansyl chromophore and one or more tryptophan residues. At a slower rate the peptide might bind to the active site to give a more specific and more stable complex of reduced fluorescence. However, in experiments where peptide is in excess, it should continue to bind nonspecifically and this is not seen. Alternatively, the peptide might initially bind loosely at the active site with the dansyl group very near a Trp residue or residues followed by a rearrangement of the complex to give the stable final complex with the dansyl group further away from the Trp(s), perhaps even more exposed to the solvent. One possibility is that the initial complex has the dansyl group deeply buried in the hydrophobic active site, i.e., the peptide binding end on to the enzyme. Rearrangement to allow the hydrophobic 4–7 sequence to bind at the active site would move the dansyl group further away. However, nondansylated peptides also exhibit to two-step kinetic pathway even though they do not have an aromatic terminus. Since the active site region of the acid proteases is partially blocked by a loop or "flap", it is conceivable that the slow step in these rearrangements might be opening of the active site to permit peptide entry. One of the referees has suggested another explanation for the decrease in fluorescence intensity. The closest Trp residue to the dansyl group might be quenched by another mechanism due to a conformational change that alters its environment. This conformational change would also lead to inhibition. This is a perfectly valid alternative that cannot be distinguished at this point. However, it is clear that any explanation must include a conformational change or a translation of some nature.

Resolution of these possibilities can best be achieved by studying the initial, very rapid binding in more detail and by placing the dansyl chromophore at different locations in the peptide chain.

The energy transfer procedure does not yield distances of sufficient precision to localize the inhibitor with any confidence (Berman et al., 1980; Dale et al., 1979; Haas et al., 1978; Marsh & Lowey, 1980; Schiller, 1972, 1981; Schiller et al., 1977; Stryer, 1978). The situation for porcine pepsin in especially difficult since these are five Trp residues in the protein and the dansyl chromophore is probably receiving some energy from all five (Zukin et al., 1977; Penny & Dyckes, 1980). However, the data do suggest that the two complexes, initial and final, differ in relative position of the dansyl chromophore of 8–9 Å.

The gel filtration experiments presented in Figure 5 also provide physical evidence for complex formation between pepsin and dansyl peptides. Our observation that pepstatin can displace the dansyl peptide can be interpreted that both species bind in a common or at least overlapping region. This is consistent with active site binding of the dansyl peptides, since pepstatin is known to bind to the active site of the acid proteases.

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Polypeptide Molecular Weights of the (Na^+,K^+)-ATPase from Porcine Kidney Medulla†

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ABSTRACT: The molecular weights of the polypeptide chains from (Na^+,K^+)-ATPase of porcine kidney medulla have been determined by analytical sedimentation equilibrium. The α-subunit molecular weight is 93 900, and the β subunit is a glycoprotein with a polypeptide molecular weight of 32 300 (41 400 including protein and carbohydrate). Amino acid and carbohydrate compositions are presented together with related properties (i.e., partial specific volumes, extinction coefficients, and hydrophobic/hydrophilic amino acid content).

Plasma membranes of animal cells contain an ion pump, (Na^+,K^+)-ATPase, that maintains the intracellular space high in potassium and low in sodium. This membrane-bound (Na^+,K^+)-ATPase transduces the energy from ATP hydrolysis to the countertransport of Na^+ and K^+ against a concentration gradient. The pump protein has been purified in membrane-bound form by a number of investigators [e.g., see Jorgensen (1974), Uesugi et al. (1971), Dixon & Hokin (1974), and Hokin et al. (1973)] and as a detergent-solubilized protein–lipid complex by Hastings & Reynolds (1979) and Esmann et al. (1979). In all purified preparations thus far investigated, two polypeptide chains are observed under denaturing conditions in sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The catalytic subunit (α) has an *apparent* molecular weight between 85 000 and 106 000 while the β polypeptide has *apparent* molecular weights varying between 36 000 and 65 000 and contains covalently bound carbohydrate.

Despite extensive kinetic studies of (Na^+,K^+)-ATPase from a wide variety of species and organs together with structural investigations of conformational changes during the ATP hydrolysis cycle, we are lacking the most fundamental information required for a mechanistic model. The absolute number of polypeptide chains in the minimal functioning unit is still a subject of controversy, and this information cannot be obtained in the absence of rigorous molecular weight determinations of the catalytic and glycoprotein subunits. Absolute molecular weights and polypeptide chain stoichiometry have been determined by sedimentation equilibrium for the (Na^+,K^+)-ATPase from dogfish shark rectal gland (Hastings & Reynolds, 1979; Esmann et al., 1979). Although both groups obtain the same molecular weights for the individual polypeptides (α = 106 000 and β = 36 000–40 000), different chain stoichiometries were found for the minimal functioning unit, $\alpha_2\beta_4$ and $\alpha_2\beta_2$, respectively. These data are not available for the mammalian enzyme, but estimates of polypeptide stoichiometry and the minimal molecular weight of a functioning unit have been published by using indirect methods [e.g., cross-linking (Craig & Kyte, 1980) and active site binding (Jorgensen, 1980)]. *Apparent* polypeptide molecular weights have been reported for the mammalian enzyme by

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