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Fluorescence Studies on the Interaction of Pepsin with Its Substrates†

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ABSTRACT: A series of pepsin substrates having an amino-terminal dansyl (Dns) group has been synthesized, and measurement of the kinetic parameters of their enzymic hydrolysis has given additional evidence for the importance of secondary enzyme–substrate interactions in determining the catalytic efficiency of pepsin. Steady-state fluorescence measurements of the binding of the dansyl group by the protein have provided estimates of the dissociation constants of the enzyme–substrate complex. Whereas, under the conditions of this study, dansylamide is bound only weakly, the dansyl group of substrates such as Dns-Gly-Gly-Phe-Phe-OEt is bound strongly, with a major contribution to the binding energy coming from the interaction of the Phe-Phe unit (the sole site of enzymic hydrolysis). During the activation of pepsinogen to pepsin in the presence of a dansyl peptide ester, the enhancement of fluorescence of the dansyl group is increased.

It has been shown that, in the action of pepsin on synthetic substrates related to Z-Gly-Gly-Phe-Phe-OP4P,[1] where the Phe–Phe bond is the only sensitive linkage, the replacement of Gly-Gly by other dipeptidyl units leads to very large changes in the catalytic efficiency of the enzyme (as measured by k_{cat}[2]) without marked changes in K_M (Sachdev and Fruton, 1970). Together with other findings on the effect of the removal of the Z group, or its replacement by the less hydrophobic Moc group, this result was taken to indicate that the Z-dipeptidyl group of the substrate interacts with the enzyme as a unit, and that the benzyl portion of the benzyloxycarbonyl group participates importantly in this interaction. The possibility was considered that the large effects observed upon changing the structure of the Z-dipeptidyl unit are a consequence of the induction, by such "secondary" interactions, of conformational changes at the catalytic site of the enzyme (Fruton, 1970). Moreover, it may be expected that the conformation of the peptide in the productive enzyme–substrate complex will be influenced by such secondary interactions so as to make the Phe–Phe bond more susceptible to enzymic cleavage. Since the three-dimensional structure of pepsin has not yet been elucidated, model building cannot be used to define the limits of conformational change in either the enzyme or the substrate when the two interact productively. For this reason, we have attempted to approach this problem by the use of the fluorescent-probe technique (Edelman and McClure, 1968; Stryer, 1968). In this communication, we report data on the interaction, with pepsin, of peptides bearing an amino-terminal dansyl group as a fluorescent probe for secondary en-

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 2485 (1966), are: Dns, dansyl or 1-dimethylaminonaphthalenesulfonyl; OP4P, 3-(4-pyridyl)propyl-1-oxy; Z, benzyloxycarbonyl; Moc, methyloxycarbonyl; ANS, 1-anilinonaphthalene-8-sulfonate; TNS, 2-*p*-toluidinylnaphthalene-6-sulfonate; TPDM, tosyl-L-phenylalanyldiazomethane. Unless otherwise noted, the abbreviated designation of amino acid residues denotes the L form.

[2] The kinetic parameters mentioned in this paper are defined by the equation $v = V_m(S)/[K_M + (S)]$ for the process

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P_1 + P_2$$

where v = initial velocity, the maximal velocity $V_{max} = k_{cat} \times$ total enzyme concentration (E), (S) = initial substrate concentration, $K_M = (k_{cat} + k_{-1})/k_1$, and $K_S = k_{-1}/k_1$.

TABLE I: Synthesis and Properties of Dansylated Peptides.

Dansylated Peptide	Yield (%)	Mp (°C)	R_F[a]	Calcd (%)				Found (%)			
				C	H	N	S	C	H	N	S
Dns-Phe-Phe-OP4P	87	80–82	0.51 (A)	68.65	6.1	8.4	4.8	68.5	6.2	8.3	4.9
Dns-Gly-Phe-Phe-OP4P	88	108–110	0.56 (A)	66.55	6.0	9.7	4.4	66.4	6.1	9.9	4.5
Dns-Gly-Gly-Phe-Phe-OP4P	86	160–162	0.38 (A)	64.8	5.95	10.8	4.1	64.9	6.1	10.9	4.0
Dns-Gly-Ala-Phe-Phe-OP4P	84	179–181	0.40 (A)	65.1	6.1	10.6	4.0	65.0	6.0	10.5	3.9
Dns-Ala-Ala-Phe-Phe-OP4P	80	219–221	0.62 (B)	65.5	6.2	10.4	4.0	65.3	6.35	10.5	3.9
Dns-Gly-Sar-Phe-Phe-OP4P	82	104–106	0.34 (A)	65.1	6.1	10.6	4.0	65.0	6.2	10.5	4.1
Dns-Gly-Pro-Phe-Phe-OP4P	77	119–121	0.37 (A)	65.9	6.3	10.25	3.9	65.7	6.4	10.4	3.8
Dns-Phe-Phe-OEt[b]	90	74–75	0.63 (A)	67.0	6.15	7.3	5.6	67.2	6.25	7.4	5.5
Dns-Gly-Phe-Phe-OEt[b]	92	177–178	0.63 (A)	64.8	6.1	8.9	5.1	65.0	6.2	8.9	5.0
Dns-Gly-Gly-Phe-Phe-OEt	90	168–170	0.53 (A)	62.9	6.0	10.2	4.6	63.0	6.2	10.0	4.55
Dns-Gly-Ala-Phe-Phe-OEt[b]	88	205–207	0.55 (A)	63.3	6.2	10.0	4.6	63.1	6.2	10.0	4.7
Dns-Gly-Pro-Phe-Phe-OEt	85	118–120	0.54 (A)	64.3	6.2	9.6	4.4	63.9	6.2	9.7	4.0
Dns-Gly-Gly-D-Phe-Phe-OEt	80	180–182	0.53 (A)	62.9	6.0	10.2	4.6	63.0	6.2	10.1	4.7
Dns-D-Phe-Phe-OEt[b]	76	74–76	0.64 (A)	67.0	6.15	7.3	5.6	66.8	6.0	7.3	5.7

[a] The letter in parentheses denotes the solvent system used (see Experimental Section). [b] These compounds were not appreciably soluble in aqueous buffers of pH 3–10.

zyme–substrate interaction. Advantage was taken of the enhancement of the fluorescence of the dansyl group, and the shift of its emission maximum to a shorter wavelength, when this group is transferred from an aqueous environment to a nonpolar environment or is bound to proteins (Weber, 1952; Lagunoff and Ottolenghi, 1966; Chen, 1967). The binding of the dansyl group of Dns-peptides to pepsin was studied at the pH value (3.1) at which data had been obtained for the kinetic parameters of their enzymic cleavage, to permit comparison of the results obtained from fluorescence and kinetic studies.

Experimental Section

Chromatography. The dansyl peptides prepared for these studies were examined chromatographically by means of Eastman Chromagram sheets 6061, with the following solvent systems: (A) ethyl acetate–methanol (9:1, v/v), and (B) chloroform–methanol (9:1, v/v). Iodine vapor and ultraviolet light were used to detect the spots.

Synthesis of Dansyl Peptides. The peptides used in these experiments were made by the reaction of the corresponding unblocked peptide esters with dansyl chloride (Aldrich Chemical Co.). The unblocked peptide esters were obtained by treatment of the corresponding Z-peptide-OP4P esters (Sachdev and Fruton, 1969, 1970) with HBr–acetic acid, or by catalytic hydrogenolysis of the corresponding Z-peptide-OEt esters in ethanol containing 1 equiv of acetic acid. The Z-peptide-OEt esters prepared as intermediates were: Z-Phe-Phe-OEt (Inouye *et al.*, 1966), Z-Gly-Phe-Phe-OEt (mp 80–82°), Z-Gly-Gly-Phe-Phe-OEt (mp 164–165°), Z-Gly-Ala-Phe-Phe-OEt (mp 148–149°), Z-Gly-Pro-Phe-Phe-OEt (mp 144–145°), and Z-Gly-Gly-D-Phe-Phe-OEt (mp 163–164°).

The dansylation was performed as follows. The unblocked peptide ester (1 mmole) was dissolved in ethanol (25 ml) and 0.2 ml of aqueous $NaHCO_3$ was added. Dansyl chloride (1.4 mmoles) in ethanol (10 ml) was added, the pH was adjusted to 9.0 by dropwise addition of freshly distilled triethylamine, and the reaction mixture was stirred for 4 hr at room temperature. The ethanol was evaporated *in vacuo*, the residue was dissolved in chloroform (5 ml), and the solution was passed through a column (1.5 × 50 cm) containing 20 g of silica gel (Baker, for chromatographic use), prepared with ethyl acetate. Elution was begun with ethyl acetate to remove the by-product dansylamide (three 10-ml fractions). Further elution with ethyl acetate–methanol (9:1, v/v) or chloroform–methanol (9:1, v/v) gave the desired product (five 10-ml fractions). The fractions were monitored by thin-layer chromatography on silica gel plates, and the product was crystallized either from ethyl acetate–hexane or from ethyl acetate–chloroform–hexane. Dansic acid (by-product) did not move out of the column under the conditions employed for eluting the product. The dansyl peptides obtained in this manner were recrystallized to constant melting point and to chromatographic homogeneity. This method appears to give higher yields than the one described by Gray and Hartley (1963); they used a mixture of acetone and aqueous $NaHCO_3$ for the dansylation of amino acids and peptides. The use of silica gel columns for the separation of the desired product from dansylamide and dansic acid is especially recommended. The yields, properties, and elementary analysis of the recrystallized dansyl peptides are given in Table I. In addition, Dns-Gly-Gly-Phe-OEt (mp 84–86°) and Dns-Gly-Gly-Phe (mp 105–106°) were prepared, with Z-Gly-Phe-Phe-OEt as an intermediate. The spectroscopic properties of the dansyl peptides are given in Table II; the absorption spectra were determined by means of a Cary Model 15 spectrophotometer.

Enzyme Preparations and Kinetic Studies. The preparations of pepsin (Worthington Biochemical Corp., lot 693-7) and of pepsinogen (lot PG 114) are the same as those used in earlier studies in this laboratory. TPDM-inhibited pepsin was prepared in the manner described by Delpierre and Fruton (1966). The kinetic parameters k_{cat} and K_M for the action of pepsin on dansyl peptide substrates were determined in the manner described previously (Sachdev and Fruton, 1969).

Fluorescence Measurements. We are indebted to Professor Lubert Stryer for permission to use the recording fluorescence spectrophotometer assembled in his laboratory, and to his associate Dr. Juan Yguerabide for guidance in its use; the

TABLE II: Spectroscopic Properties of Dansyl Peptides.[a]

Compound	Absorption		Fluorescence[b]	
	λ_{max} (nm)	$\epsilon \times 10^{-3}$ (M^{-1} cm^{-1})	λ_{max} (nm)	Rel Intensity[c]
Dns-Phe-Phe-OP4P	251	9.2		
	289	6.25		
	320	2.5	538 (588)	0.28
Dns-Gly-Phe-Phe-OP4P	250	10.5		
	288	6.5		
	320	2.75	534 (582)	0.33
Dns-Gly-Gly-Phe-Phe-OP4P	251	12.2		
	287	5.7		
	320	2.9	536 (583)	0.27
Dns-Gly-Gly-Phe-Phe-OEt	(247)[d]	5.3		
	285	5.9		
	320	2.4	534 (584)	0.43
Dns-Gly-Ala-Phe-Phe-OP4P	248	12.75		
	287.5	5.5		
	320	2.9	530 (584)	0.32
Dns-Ala-Ala-Phe-Phe-OP4P	248.5	12.95		
	287.5	5.3		
	320	3.25	534 (582)	0.32
Dns-Gly-Sar-Phe-Phe-OP4P	250	12.5		
	286	6.3		
	320	3.25	528 (584)	0.31
Dns-Gly-Pro-Phe-Phe-OP4P	250	12.5		
	285	5.6		
	320	3.1	536 (582)	0.27
Dns-Gly-Gly-Phe	246	5.56		
	286	6.14		
	320	2.82	538 (586)	0.28

[a] pH 3.1 (0.1 M formate), 25°. [b] Uncorrected emission maxima; values in parentheses refer to corrected emission maxima. [c] In arbitrary fluorescence units per micromolar (at uncorrected emission maximum) normalized with ANS in MeOH as standard. [d] Shoulder.

details of the instrument and its spectral response curve will be described by them in a separate communication.

In the work reported here, the excitation wavelength was 320 nm in all cases, and emission spectra were determined over the range 400–600 nm; the photomultiplier tube in the instrument was not adapted for longer wavelengths. The maxima of the emission spectra (uncorrected for the spectral response of the detection system) of the dansyl peptides are given in Table II, together with the calculated maxima of the corrected spectra; the correction factors were kindly provided by Dr. Yguerabide. In Table II are also given data for the relative fluorescence intensity (in arbitrary fluorescence units per micromole per liter) at the uncorrected emission maxima. Within the concentration range of complete solubility in the buffer system employed, the fluorescence intensity of the dansyl peptides was proportional to concentration. In all experiments reported here, the emission spectrum of a 0.003 mM solution of ANS in methanol (uncorrected emission maximum at 480 nm) was used as a standard, and the relative fluorescence intensity in separate experiments was corrected for fluctuations in the response of the instrument to the fluorescence of this compound; the absolute quantum yield of ANS in MeOH is 0.22 (Stryer, 1965). Since complete corrected emission spectra for the dansyl peptides could not be obtained, because of the sensitivity limit of the photomultiplier tube near 600 nm, absolute quantum yields for the fluorescence of these compounds cannot be reported at this time.

Photostability of Dansyl Peptides. In view of the report by D'Souza *et al.* (1970), it should be noted that there was no evidence of photolysis of the dansyl peptides under the conditions of our studies, even after exposure of an aqueous solution to the xenon lamp of the spectrophotometer (4.6×10^{13} photons cm^{-2} sec^{-1}) for 30 min; examination of the solution by the automatic ninhydrin method and by thin-layer chromatography failed to show the presence of ninhydrin-reactive components in such irradiated solutions. Nevertheless, stock solutions of the dansyl compounds (in 0.3 M formic acid) were routinely protected from light.

Binding Studies. All experiments were conducted at 25°, and the cell compartment was maintained at this temperature by means of a thermostat. Both the initial concentrations of pepsin and of dansyl peptide were varied, and the emission spectrum was scanned within 60–90 sec after the enzyme solution had been mixed with the buffered peptide solution. Because of the sensitivity of several of the dansyl peptides listed in Table I to peptic hydrolysis, only the more resistant ones could be used for steady-state fluorescence measurements of their binding to pepsin. To estimate the dissociation constant

TABLE III: Kinetics of Pepsin Action at Phe–Phe Bond of Dansylated Peptide Substrates.[a]

Dansylated Peptide	Substrate (mM)[b]	Enzyme (μM)	k_{cat} (sec^{-1})	K_M (mM)	k_{cat}/K_M (sec^{-1} mM^{-1})
Dns-Phe-Phe-OP4P	0.02–0.20 (8)	7.14	0.00134 ± 0.0003	0.04 ± 0.02	0.032
Dns-Gly-Phe-Phe-OP4P	0.02–0.20 (12)	0.089	0.42 ± 0.03	0.12 ± 0.02	3.5
Dns-Gly-Phe-Phe-OP4P[c]	0.02–0.20 (17)	0.088	0.19 ± 0.03	0.07 ± 0.02	2.7
Dns-Gly-Gly-Phe-Phe-OP4P	0.026–0.26 (11)	0.00685	5.8 ± 2.4	0.05 ± 0.02	113
Dns-Gly-Gly-Phe-Phe-OEt	0.009–0.06 (9)	0.044	0.39 ± 0.2	0.07 ± 0.04	5.7
Dns-Gly-Ala-Phe-Phe-OP4P	0.023–0.23 (8)	0.00061	31 ± 6.28	0.05 ± 0.03	574
Dns-Gly-Sar-Phe-Phe-OP4P	0.021–0.21 (11)	0.12	0.6 ± 0.15	0.31 ± 0.10	1.9
Dns-Gly-Sar-Phe-Phe-OP4P[c]	0.02–0.20 (12)	0.12	0.3 ± 0.05	0.25 ± 0.05	1.2
Dns-Gly-Pro-Phe-Phe-OP4P	0.022–0.22 (9)	6.99	0.0036 ± 0.0003	0.07 ± 0.02	0.05
Dns-Ala-Ala-Phe-Phe-OP4P	0.02–0.20 (11)	0.00027	130.6 ± 43.4	0.18 ± 0.10	726

[a] pH 3.1 (0.1 M formate buffer), 37° (unless otherwise noted). [b] The numbers in parentheses denote the number of runs. [c] 25°.

of the pepsin–peptide complex from the enhancement of fluorescence upon binding of the dansyl group to pepsin, use was made of the procedures of Weber and Young (1964) and Deranleau and Neurath (1966). A wavelength was chosen at which the uncorrected fluorescence intensity of the unbound peptide was negligible (500 nm), and the total protein concentration (P_0) was varied at constant peptide concentration (A_0). A plot of $1/F$ *vs.* $1/P_0$ gave an estimate of F_{max}, where F is the observed uncorrected fluorescence intensity (in normalized arbitrary units) and F_{max} is the estimated intensity when all of the peptide is bound to protein. From the fraction of peptide bound ($x = F/F_{max}$), the concentration of unbound protein (P) was calculated for each value of P_0, on the assumption that pepsin contains a single binding site that interacts with the Dns-peptide much more strongly than do other binding sites. A plot of x/P *vs.* x gave a straight line with an intercept near 1.0 on the x axis, and an intercept on the x/P axis corresponding to the estimated value of the association constant K in the process A + P = AP; for comparison with the K_M values derived from the kinetic data, the binding constants are given as $1/K = K_D$, the dissociation constant of the protein–peptide complex.

Results

The kinetic parameters for the action of pepsin (at pH 3.1) on a series of dansyl peptide esters are given in Table III; in all cases, chromatographic examination of the hydrolysates showed that the Phe–Phe bond of these substrates was the only one cleaved to a detectable extent under the conditions of these studies. It will be noted in Table III that the values of k_{cat}/K_M for substrates of the type A-Phe-Phe-B vary over a very large range (about 24,000-fold) depending on the nature of the A and B groups. In particular, it will be seen that when the A group is lengthened by the addition of one or two glycyl residues (A = Dns, Dns-Gly, or Dns-Gly-Gly), the value of k_{cat} is greatly increased, without much change in the value of K_M. It may be concluded, therefore, that as in the case of the corresponding series where A = Z, Z-Gly, or Z-Gly-Gly (Sachdev and Fruton, 1969), the secondary interactions of the amino-terminal blocking group with the enzyme has a large effect on the catalytic efficiency in the cleavage of the Phe–Phe bond. It is noteworthy that replacement of the Z group by the Dns group causes a marked decrease in both k_{cat} and K_M, especially in the case of Dns-Phe-Phe-OP4P. Among the possible explanations for this effect is a greater contribution to nonproductive interaction with the dansyl compound.

It is of special interest that for the series of substrates where the A group is a Dns-dipeptidyl unit, changes in the amino acid residues composing this unit (Gly-Gly, Gly-Ala, Gly-Sar, Gly-Pro, or Ala-Ala) have a very large effect on k_{cat}, and that the k_{cat}/K_M values increase in the same order as for the corresponding series of substrates having a Z-dipeptidyl unit (Sachdev and Fruton, 1970). Except for the substrate with A = Gly-Sar, the k_{cat} values are about one order of magnitude less than those for analogous substrates having a Z-terminal group. These results indicate that the Dns-dipeptidyl unit interacts with pepsin, and that the amino acid residues separating the dansyl group from the sensitive Phe–Phe bond have a considerable effect on catalytic efficiency. In its secondary interaction with pepsin, therefore, the Dns-dipeptidyl portion of the substrate appears to act as a unit, in a manner comparable to that suggested previously for the corresponding Z-peptide-OP4P esters (Sachdev and Fruton, 1970). It will be noted that for the pair of substrates Dns-Gly-Gly-Phe-Phe-B, where B = OP4P or OEt, the K_M values are nearly the same but that the pyridyl ester is cleaved at the Phe–Phe bond about 15 times more rapidly than is the ethyl ester; a similar ratio of k_{cat} values was found for the OP4P and OEt esters of Gly-Gly-Phe-Phe (Sachdev and Fruton, 1969; Hollands *et al.*, 1969). It would appear that, in the case of these two pairs of substrates, the contribution of the B group to enzyme–substrate interaction is independent of the nature of the A group; similar findings have been reported for other pepsin substrates (Medzihradszky *et al.*, 1970).

To study the binding, to pepsin, of several of the Dns-peptide esters listed in Table I, the fluorescence experiments were conducted at pH 3.1 (0.1 M formate buffer) and 25°; consequently, in the binding studies the enzyme was in the same ionic state as in the kinetic studies, except for the difference in temperature. At this pH value, the dansyl group of free dansylamide is largely in the protonated form, since the pK_a of this compound has been estimated to be near pH 3.9 (Lagunoff and Ottolenghi, 1966). It may be expected that the dansyl group of the Dns-peptide esters has a similar pK_a value. The solubility of the ethyl esters in aqueous solution is largely a consequence of the protonation of the dansyl group and, as noted in Table I, several of these compounds proved to be in-

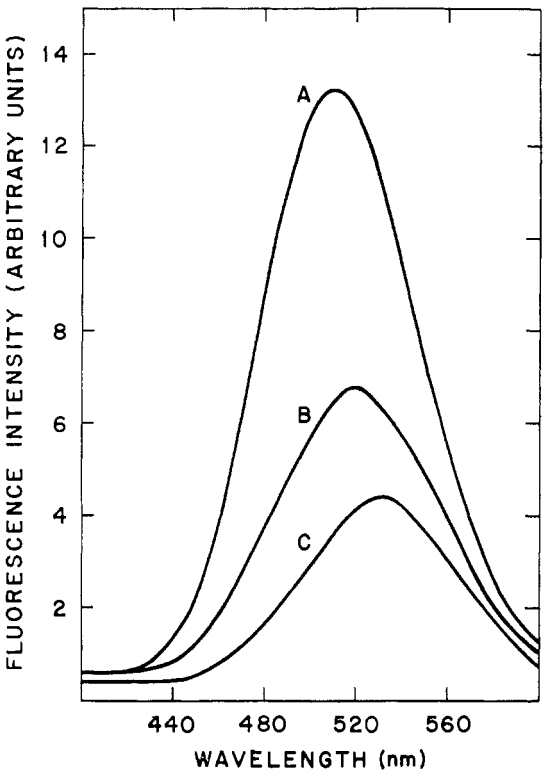

FIGURE 1: Emission spectra of Dns-Gly-Pro-Phe-Phe-OP4P. Substrate concentration, 15.6 μM; pH 3.1 (0.1 M formate buffer); 25°. The spectra are uncorrected. Curve A, with pepsin, 12.6 μM; curve B, with TPDM-pepsin, 12.6 μM; curve C, no pepsin.

sufficiently soluble for use in kinetic or fluorescence studies. The OP4P esters were more soluble, owing to the presence of the pyridinium group.

As shown by Lagunoff and Ottolenghi (1966), although the absorption spectrum of the protonated dansylamide (in 60% ethanol, at pH 1.0) is very different from that of the conjugate base (at pH 9.75), the latter showing increased absorbance at 320 nm, the corrected emission maximum at 580 nm (excitation at 285 nm) is the same at pH 2.1 and 7.1. It will be seen in Table II that, under the conditions of our studies, the Dns-peptide esters gave corrected emission maxima in the range 582–588 nm. It is of interest that whereas the relative fluorescence intensity at the uncorrected emission maximum for the Dns-peptide-OP4P esters was nearly the same (0.27–0.33 arbitrary unit/μmole per l.), replacement of the protonated pyridiniumpropyl group of Dns-Gly-Gly-Phe-Phe-OP4P by an ethyl group led to a marked increase in fluorescence intensity. In this connection, it may be noted that Chen (1967) has reported that, in aqueous buffer at pH 7.0, the corrected emission maxima for various dansylamino acids are at 578–580 nm (excitation at 335 nm) but that the fluorescence quantum yields differ widely, depending on the nature of the amino acid.

The fact that there is a large difference in the binding of dansylamide or Dns-Phe to pepsin, as compared with the interaction of the enzyme with a good substrate is shown in Table IV. There was no detectable change in fluorescence when dansylamide (13.9 μM) was mixed with pepsin (11.1 μM), and even at protein concentrations as high as 150 μM, the fluorescence of 13.9 μM dansylamide was only slightly enhanced, indicating very poor binding of the dansyl group by pepsin in an aqueous medium of pH 3.1. As noted in Table IV, Dns-Phe was bound somewhat more strongly, but the addition of only 0.3 μM pepsin to the substrate Dns-Gly-Gly-Phe-Phe-OEt produced a large increase in fluorescence intensity and a significant shift to a shorter wavelength. Within 2 hr, the emission spectrum of the enzyme–substrate mixture had changed to that given by an equivalent solution of Dns-Gly-Gly-Phe, one of the cleavage products in the pepsin-catalyzed reaction. This compound is bound to pepsin (11.1 μM) more strongly than either dansylamide or Dns-Phe (see Table IV), but at the lower enzyme concentration used with Dns-Gly-Gly-Phe-Phe-OEt no binding of Dns-Gly-Gly-Phe to pepsin could be detected by fluorescence measurements. Stopped-flow kinetic studies of fluorescence changes during the pepsin-catalyzed cleavage of sensitive Dns-peptide substrates are in progress, and will be reported in a separate communication.

TABLE IV: Interaction of Pepsin with Dansyl Compounds.[a]

Compound	No Pepsin		With Pepsin[b]	
	λ_{max} (nm)	I	λ_{max} (nm)	I
Dansylamide	530	4.5	530	4.5
Dns-Phe	530	1.7	528	2.1
Dns-Gly-Gly-Phe	540	3.8	532	5.4
Dns-Gly-G y-Phe-Phe-OEt	534	6.0	528[c]	7.8[c]

[a] Concentration of dansyl compound, 13 μM; pH 3.1 (0.1 M formate buffer); 25°. The emission maxima are uncorrected, and the relative fluorescence intensity (I, in arbitrary units) is normalized with ANS in methanol as a standard. [b] Except as noted, the pepsin concentration was 11.1 μM. [c] Pepsin concentration, 0.3 μM; fluorescence measured within 60 sec of mixing enzyme and substrate.

With more resistant peptides, such as Dns-Gly-Pro-Phe-Phe-OP4P (*ca.* 10 μM), the initial enhancement of fluorescence upon the addition of pepsin is invariant during the first 90 sec after the addition of as much as 100 μM enzyme, and estimates can be made of the binding of the dansyl group to the protein. In Figure 1 are shown typical emission spectra for this substrate in the presence of added pepsin; under the conditions of this experiment, with an approximately 1:1 molar ratio of peptide to protein, there was a shift in the uncorrected emission maximum of about 18 nm, and a nearly threefold increase in the fluorescence intensity at the maximum. From a series of experiments in which the pepsin concentration was varied, the value of K_D was estimated to be 0.04 mM. This value of K_D (25°) is similar to the value of K_M (37°) reported in Table III; it will be noted from the table that for the two substrates whose kinetic parameters were determined both at 25 and 37°, the values of K_M did not change markedly over this temperature range, whereas k_{cat} was approximately twice as high at the higher temperature.

That the binding of the dansyl group of Dns-Gly-Pro-Phe-Phe-OP4P depends in large part on the interaction of the Phe-Phe-OP4P portion of the substrate with the catalytic site of the enzyme is shown in Figure 1 by the result with pepsin that had been inactivated by the active-site-directed inhibitor tosyl-L-phenylalanyldiazomethane (Delpierre and Fruton, 1966). With the same ratio of pepsin to peptide as for the experiment with untreated enzyme, the enhancement of fluores-

cence was greatly decreased. From experiments in which the protein concentration was varied, the value of K_D (25°) for the interaction of the dansyl group of Dns-Gly-Pro-Phe-Phe-OP4P with TPDM-pepsin was estimated to be 0.13 mM.

With another relatively resistant substrate, Dns-Phe-Phe-OP4P, fluorescence measurements gave an estimated value of K_D (25°) of 0.13 mM. It will be noted from Table III that the observed value of K_M for the cleavage of this substrate by pepsin at 37° was somewhat lower than the K_D value. The K_D values for the Dns-peptide substrates that are hydrolyzed more rapidly by pepsin could not be estimated reliably from steady-state fluorescence measurements.

It has been shown (Inouye and Fruton, 1967) that the replacement, by its D enantiomer, of either L-phenylalanyl residue of the sensitive Phe-Phe unit of a pepsin substrate renders the Phe–Phe bond resistant to enzymic action. Such diastereoisomeric peptides are competitive inhibitors of pepsin, and their K_I values are similar to the K_M values for the corresponding L,L substrates. It will be noted from Table III that for Dns-Gly-Gly-Phe-Phe-OEt, the estimated value of K_M is 0.07 mM at 37°. Fluorescence measurements of the binding of the diastereoisomeric Dns-Gly-Gly-D-Phe-Phe-OEt to pepsin at 25° gave an estimated value of K_D of 0.04 mM.

The interaction of Dns-Gly-Pro-Phe-Phe-OP4P with pepsinogen initially gave a smaller enhancement of fluorescence than that observed with an equivalent amount of pepsin. Under the conditions of the experiments described in Figure 2, the fluorescence increased with time, and reached a maximum value within 20 min, corresponding to the activation of the zymogen.

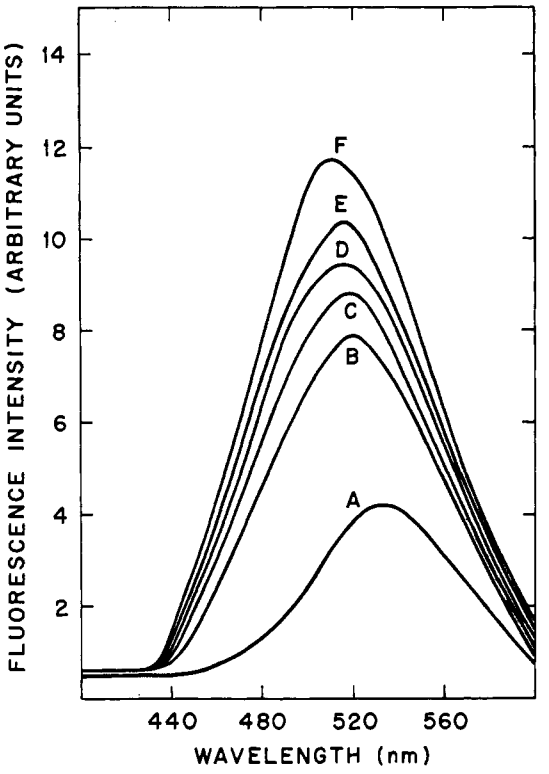

FIGURE 2: Emission spectra of Dns-Gly-Pro-Phe-Phe-OP4P. Substrate concentration, 13.9 μM; pepsinogen concentration, 12.9 μM; pH 3.1 (0.1 M formate buffer); 25°. The spectra are uncorrected. Curve A, no pepsinogen; curves B to F, 2, 4, 6, 8, and 20 min, respectively, after addition of pepsinogen.

Discussion

It is evident from the data presented above that under conditions where the dansyl group of Dns-peptide substrates is bound strongly to pepsin, dansylamide is bound weakly, if at all. The striking increase in the affinity of pepsin for the dansyl group when it is part of a peptide such as Dns-Gly-Gly-Phe-Phe-OEt indicates that the specific interaction of the Phe-Phe-OEt portion of the substrate with the active site of the enzyme contributes significantly to the binding of the dansyl group. Previous studies on the inhibition of pepsin by dipeptide esters such as Phe-Phe-OEt have given K_I values near 0.2 mM (pH 4, 37°), closely approximating the K_M values for substrates such as Z-His-Phe-Phe-OEt (Inouye and Fruton, 1968). It may be suggested, therefore, that the dansyl group of a substrate such as Dns-Gly-Gly-Phe-Phe-OEt is "dragged" into a hydrophobic region of the enzyme whose intrinsic affinity for the dansyl group may be low. The marked increase in the apparent dissociation constant for the interaction of the dansyl group of Dns-Gly-Pro-Phe-Phe-OP4P with TPDM-pepsin, where the catalytic site has been stoichiometrically blocked with a tosyl-L-phenylalanylmethyl group, is consistent with this suggestion. It should be added that, in the presence of excess Phe-Phe-OP4P, there was no detectable increase in the binding of dansylamide by pepsin at pH 3.1.

It is significant that with a resistant Dns-peptide ester having a D-phenylalanyl residue (Dns-Gly-Gly-D-Phe-Phe-OEt), the K_D value for the binding of the dansyl group to pepsin is similar to the K_M value for the comparable diastereoisomeric substrate Dns-Gly-Gly-Phe-Phe-OEt. This result supports the view that the dansyl group of sensitive substrates is "dragged" into the enzyme by the specific interaction of the Phe-Phe unit of such substrates. When this unit is replaced by the diastereoisomeric D-Phe-Phe unit, the latter is bound at the catalytic site of pepsin in a conformation that renders the peptide bond resistant to enzymic attack, but the extent of the interaction of the amino-terminal dansyl group with a hydrophobic region of the protein appears to be unaffected.

These results raise the possibility that other proteinases may exhibit a similar behavior. Deranleau and Neurath (1966) found that chymotrypsin and chymotrypsinogen bind dansylamide and Dns-Gly-OEt relatively weakly (K_D = 2 mM) at pH 7.8, but that the competitive inhibitor Dns-D-Trp-OEt is bound more strongly (K_D = 0.5 mM). Chen (1967) has reported that various dansylamino acids are bound weakly to chymotrypsin and chymotrypsinogen at pH 7.0, in contrast to the strong interaction with bovine serum albumin and apomyoglobin. That serum albumin has exceptional affinity for fluorescent probes for hydrophobic sites has been demonstrated by Weber (1952), in his studies with ANS, and Stryer (1965) has shown that ANS is tightly bound by apomyoglobin in the hydrophobic region occupied by the heme portion of myoglobin. Moreover, Chen and Kernohan (1967) have reported that dansylamide is tightly bound by carbonic anhydrase at pH 7.4 (K_D = 0.26 μM). In the case of chymotrypsin and chymotrypsinogen, McClure and Edelman (1967) have found that TNS is bound at pH 7.8 with K_D values in the range 0.2–0.5 mM; they concluded that TNS is bound at a region of the enzyme outside the catalytic site. It will be of interest, therefore, to examine the interaction of the dansyl group of Dns-peptide substrates of chymotrypsin with this enzyme in a manner similar to that described here for pepsin.

The finding that the interaction of pepsinogen with Dns-Gly-Pro-Phe-Phe-OP4P leads to a smaller enhancement of fluorescence than that observed with pepsin is consistent with

the suggestion that the locus of binding of the dansyl group is at a distance from the catalytic site, and also indicates that the conformational change in the activation of the zymogen promotes such binding. Wang and Edelman (1971) have provided strong evidence in favor of the view that TNS is bound by pepsinogen and pepsin at a locus distinct from catalytic site of the enzyme; at pH 4.5, the binding was characterized by a K_D of 0.2–0.3 mM. They observed that the maximal fluorescence of the TNS–pepsin complex was only 35% of that for the TNS–pepsinogen complex, and that upon activation of the zymogen in the presence of TNS the fluorescence intensity decreases. Further studies are needed to explain the difference in the fluorescence behavior of the Dns-peptide substrates and of TNS during the activation process, but it appears likely that the difference lies in the role of the interaction of the Phe-Phe portion of the substrates with the catalytic site of the active enzyme. Also, the possibility exists that the binding site in pepsinogen and pepsin for TNS may be different from the one for the dansyl group.

Steady-state fluorescence measurements could not be used to estimate the binding constants for the interaction of the dansyl group of sensitive Dns-peptide substrates, because of their rapid cleavage of the Phe–Phe bond by pepsin. With two relatively resistant substrates (Dns-Gly-Pro-Phe-Phe-OP4P and Dns-Phe-Phe-OP4P) the ratio of K_D values (0.04:0.13 mM) was approximately inversely proportional to the ratio of k_{cat} values (0.0036:0.0013 sec^{-1}). Although this result suggests that tighter binding of the dansyl group may be associated with higher catalytic efficiency, no definite conclusion on this question can be offered on the basis of the data at hand. Clearly, stopped-flow fluorescence measurements with dansyl peptide esters of widely varying sensitivity to pepsin are needed.

In the face of the limited knowledge regarding the three-dimensional structure of pepsin, speculations regarding the precise conformation of a pepsin substrate when it forms part of a productive enzyme–substrate complex are premature. In particular, it is not yet possible to make reliable estimates of the distances between the catalytic site of pepsin and the loci of secondary enzyme–substrate interaction, as had been done in the case of carboxypeptidase by Latt *et al.* (1970). The kinetic data presented in this and other communications indicate, however, that such secondary interactions play an important role in determining the catalytic efficiency of pepsin, as well as other enzymes that act on oligomeric substrates (Fruton, 1970, 1971); moreover, the data offered above provide additional support for the view that some of the features of these secondary interactions are accessible to fruitful study by fluorescence measurements.

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