N-Methyl-2-anilinonaphthalene-6-sulfonyl Peptides as Fluorescent Probes for Pepsin-Substrate Interaction*

(Received for publication, March 11, 1973)

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SUMMARY

Advantage has been taken of the high sensitivity of the N-methyl-2-anilinonaphthalene-6-sulfonyl (mansyl) group as a fluorescent probe for hydrophobic regions of proteins to study the interaction of pepsin with mansylamide and with several peptides bearing an amino-terminal mansyl group. The data indicate that pepsin has a binding site for mansylamide that is distinct from the substrate-binding region of the enzyme. When a mansyl group is attached to a peptide having an L-phenylalanyl-L-phenylalanyl bond, previously identified as a preferred linkage for attack by pepsin, the fluorescent probe appears to be drawn into a hydrophobic region not evident in the interaction with mansylamide. Upon the addition of pepstatin, a powerful competitive inhibitor of pepsin, the enhancement of the fluorescence of mansylamide is unaffected, but that of the mansyl peptides is reduced to the value for mansylamide. Further evidence for the discrete nature of the binding sites in pepsin for mansylamide and for the mansyl group of peptide substrates is provided by the fact that, upon autocatalytic activation of pepsinogen, the enhancement of the fluorescence of mansylamide is decreased, whereas that of the mansyl peptides is increased; both effects are abolished by pepstatin.

Studies on the fluorescence of mansylamide and of maneryl peptides in the presence of pepsin that had been stoichiometrically inactivated by treatment with tosyl-L-phenylalanyldiazomethane have shown that this active site-directed inhibitor not only blocks the access of a mansyl peptide substrate to the active site, but also alters the mansylamide-binding site so as to lower its polarity. Further evidence for the conformational flexibility of pepsin suggested by this finding is provided by data on the partially acetylated enzyme. When a mansyl group is attached to a peptide having an L-phenylalanyl-L-phenylalanyl bond, previously identified as a preferred linkage for attack by pepsin, the fluorescence of the dansyl group (provided as dansylamide) at pH values near 3. However, when this group is part of a pepsin substrate such as Dns-Gly-Pro-Phe-Phe-OP4P, which is cleaved exclusively at the Phe-Phe bond, an enhancement of the fluorescence intensity and a shift in the emission maximum of the dansyl group are clearly evident. This interaction of the dansyl group with the protein suggests that it had been “dragged” into a region of the enzyme surface characterized by a lower polarity than that of the aqueous solution. Moreover, this secondary interaction of the amino-terminal substituent is associated with marked effects on the catalytic efficiency of pepsin (as measured by kcat) without significant changes in the values for Km.

Data are presented for the kinetics of the cleavage by pepsin of several mansyl peptide substrates. These results, together with those from the fluorescence studies, give further evidence of the importance of secondary interactions of pepsin substrates with the extended active site of the enzyme in influencing catalytic efficiency without marked change in total binding energy. It is difficult to accommodate the findings reported here with a relatively rigid extended active site that can be mapped in terms of subsites; instead, pepsin appears to exhibit considerable conformational flexibility at the active site in response to enzyme-substrate (or enzyme-inhibitor) interaction.

Previous work in this laboratory has shown that pepsin substrates bearing an amino-terminal dansyl1 group are useful for fluorescence studies on the secondary interactions of peptides with pepsin (1). Steady state fluorescence measurements showed that pepsin does not have appreciable intrinsic affinity for the dansyl group (provided as dansylamide) at pH values near 3. However, when this group is part of a pepsin substrate such as Dns-Gly-Pro-Phe-Phe-OP4P, which is cleaved exclusively at the Phe-Phe bond, an enhancement of the fluorescence intensity and a shift in the emission maximum of the dansyl group are clearly evident. This interaction of the dansyl group with the protein suggests that it had been “dragged” into a region of the enzyme surface characterized by a lower polarity than that of the aqueous solution. Moreover, this secondary interaction of the amino-terminal substituent is associated with marked effects on the catalytic efficiency of pepsin (as measured by kcat) without significant changes in the values for Km.

1 The abbreviations used are: dansyl or Dns, N,N-dimethyl-l-anilinonaphthalene-5-sulfonil; OP4P, 3-(4-pyridyl)propyl-1-oxyl; Z, benzoyloxycebutonyl; mansyl or Mns, N-methyl-2-anilinonaphthalene-6-sulfonate; OEt, ethoxy; TPDM, tosyl-L-phenylalanyldiazomethane; TNS, 2-p-toluidino-5-(3-methoxy-4-nitro)phenylalanine; Phe(NO)2, p-nitro-L-phenylalanyl. The abbreviated designation of amino acid residues denotes the L form.

* This study was aided by Grants GM-18172 and AM-15683 from the National Institutes of Health and by Grant GM-18268 from the National Science Foundation.
† This paper is offered by J. S. F. in token of friendship to Professor Theodor Wieland, on the occasion of his sixtieth birthday.

V, No. 18, Issue of September 25, pp. 6292-6299, 1973
Printed in U.S.A.
binding of mansylamide by bovine serum albumin. Sodium mansate was used by Birkett to describe the synthesis of mansyl chloride and reported on the hydrophobic probe was proposed by Cory et al. (3), who demonstrated the importance of the interaction of the A group (including its amino-terminal substituent) with pepsin in affecting the rate at which the Phe-Phe bond is hydrolyzed (2).

In extending our fluorescence studies, we have examined the behavior of other derivatives of aminonaphthalenesulfonic acid as fluorescent probes for the secondary interaction of pepsin with its substrates. In this communication, we report results obtained with compounds bearing an N-methyl-2-anilinonaphthalene-6-sulfonyl (mansyl) group. The use of this group as a fluorescent probe for the secondary interaction of pepsin with its substrates was examined for their homogeneity by thin layer chromatography. The residue was then dissolved in ethanol (35 ml) and 0.2 ml of aqueous NaHCO3 was added to the resulting solution. The mixture was stirred for 5 hours at room temperature. The solution was then cooled to room temperature and poured over crushed ice to yield a yellow oil, which crystallized readily. The product was washed with ice-cold water, dried in air, and extracted with anhydrous ether (50 ml). After being filtered, the ethereal solution was concentrated to yield 1.9 g (95%) of the product, m.p. 130-131°C. Cory et al. (3) reported a melting point of 128-133°C and 44% yield. Chromatography (Solvents A and C) gave single spots of RF, 0.68 and 0.61, respectively.

The mansyl derivatives used in this work were prepared in a manner similar to that described previously (1) for the dansylation of peptides. The blocked peptide esters were obtained by treatment of the corresponding Z-peptide-OEt or Z-peptide-OP4P esters with HBr-acetic acid (1, 2, 5). The unblocked amino acid ester or peptide ester hydrobromide (1 mmole) was dissolved in ethanol (35 ml) and 0.2 ml of aqueous NaHCO3 (5%) was added. Mansyl chloride (1 mmole) dissolved in acetone (10 ml) was added, the pH was adjusted to 0.0 by dropwise addition of freshly distilled triethylamine, and the reaction mixture was stirred for 5 hours at room temperature. The solvent was evaporated in vacuo and the residue was examined by thin layer chromatography. The residue was then dissolved in 5 ml of chloroform-ethyl acetate (1:1, v/v) or CH2Cl2 (5 ml), and the solution was passed through a column (1.2 x 50 cm) containing 20 g of silica gel (Baker, 60 to 200 mesh) prepared with ethyl acetate. The column was then eluted with ethyl acetate, and 5-ml fractions were collected after the appearance of the first fluorescent fraction. The first three fractions usually had a fluorescent impurity (mansylamide) and were well separated from the products. Further elution with ethyl acetate gave the desired product. Mansic acid (byproduct) did not move out of the column under the conditions employed for eluting the product. The yields, properties, and elementary analyses of the recrystallized mansyl compounds are given in Table I; their spectroscopic properties are reported in Table II. The absorption spectra were determined by means of a Cary model 15 spectrophotometer. Because of the limited solubility of the

Table I

| Table I | Synthesis and properties of mansyl compounds |
|---------|---------------------------------------------|
| Mansyl Compounds | Yield (%) | Mp (°C) | Rf | C | H | N | S | C | H | N | S |
| Mansyl-H2 | 95 | 158-159 | 0.61 (A) | 65.5 | 5.2 | 9.0 | 10.3 | 65.3 | 5.4 | 8.8 | 10.1 |
| Mansyl-Phe-OEt | 77 | 143-144 | 0.65 (A) | 68.8 | 5.8 | 5.7 | 6.6 | 69.0 | 5.7 | 5.6 | 6.8 |
| Mansyl-Phe | 87 | 152-154 | 0.74 (B) | 67.8 | 5.3 | 6.1 | 7.0 | 68.0 | 5.2 | 6.0 | 6.8 |
| Mansyl-Gly-Phe-OEt | 91 | 140-141 | 0.65 (A) | 66.0 | 5.7 | 7.8 | 5.9 | 65.9 | 5.5 | 7.0 | 6.0 |
| Mansyl-Gly-Phe | 57 | 152-153 | 0.68 (B) | 65.0 | 5.3 | 8.1 | 6.2 | 64.8 | 5.6 | 8.0 | 6.3 |
| Mansyl-Gly-Gly-Phe-OEt | 85 | 157-159 | 0.63 (A) | 63.8 | 5.7 | 9.3 | 5.3 | 64.1 | 5.5 | 9.1 | 5.1 |
| Mansyl-Gly-Gly-Phe | 88 | 169-171 | 0.56 (D) | 62.7 | 5.3 | 9.8 | 5.6 | 62.6 | 5.4 | 9.7 | 5.7 |
| Mansyl-Gly-Gly-Phe-OP4P | 70 | 89-91 | 0.50 (A) | 71.1 | 5.8 | 7.7 | 4.4 | 70.9 | 6.0 | 7.6 | 4.4 |
| Mansyl-Gly-Gly-Phe-OP4P | 76 | 126-128 | 0.48 (A) | 68.9 | 5.8 | 8.9 | 4.1 | 69.1 | 5.7 | 9.1 | 3.9 |
| Mansyl-Gly-Gly-Phe-OP4P | 48 | 108-110 | 0.46 (A) | 67.1 | 5.8 | 10.0 | 3.8 | 67.0 | 5.9 | 9.8 | 4.0 |

* The letter in parentheses denotes the solvent system used (see “Experimental Procedure”).
* Cory et al. (3) reported a 53% yield of a material melting at 157-159°C.
* These compounds were not appreciably soluble in aqueous formic acid (0.5 M), pH 2.35.

where v = initial velocity, the maximal velocity Vmax = kmax X total enzyme concentration, E, (S) = initial substrate concentration, Ks = (kcat + kcat)/kcat, and kcat = kcat/kcat. The dissociation constant of the complex formed between pepsin and a ligand is denoted Kd.
TABLE II
Spectroscopic properties of mansyl and dansyl compounds in aqueous solution

Spectra were determined at pH 2.35 (0.5 M formic acid) and 25°C; each compound was at 10 μM concentration. Fluorescence was measured at an excitation wave length of 335 nm for mansyl compounds and 324 nm for dansyl compounds.

| Compound              | Absorption (nm) | Fluorescence (nm) |
|----------------------|----------------|-------------------|
|                      | λ_{max} (nm)  | ε × 10^4 | λ_{max} (nm) | Intensity |
| Mns-NH₂              | 324           | 1.78     | 450         | 0.03      |
| Dns-NH₂              | 284           | 0.85     | 540         | 0.05      |
| Mns-Phe-Phe-OP₄P     | 255           | 2.54     | 480         | 0.06      |
| Dns-Phe-OP₄P         | 254           | 0.75     | 318         | 0.70      |
| Mns-Gly-Phe-OP₄P     | 255           | 2.54     | 390         | 0.05      |
| Mns-Gly-Gly-Phe-OP₄P| 324           | 1.78     | 318         | 0.25      |

* Fluorescence intensity at uncorrected emission maximum, given in arbitrary units (normalized with quinine sulfate).

manysyl compounds in aqueous solvents, it was necessary to prepare stock solutions in 5 M formic acid; aliquots were diluted 10-fold in making up incubation mixtures for kinetic and fluorescence studies at pH 2.35. The absorbance and fluorescence of the mansyl and dansyl compounds tested were proportional to concentration, indicating that no measurable aggregation was occurring under the conditions of our studies. The compounds and their solutions were protected from light prior to their use in fluorescence experiments.

**Enzyme Preparations and Kinetic Studies**—The preparations of pepsin (Worthington Biochemical Corp., Lot 693-7) and of pepsinogen (Worthington, Lot PG 114) are the same as those used in earlier studies in this laboratory. TPTM-inhibited pepsin and alkali-inactivated pepsin (pH 7.8) were prepared in the manner described by Delpierre and Fruton (6). Acetylpepsin (containing 4 acetyl groups per molecule of protein) was prepared as described by Hollands and Fruton (7). The kinetic parameters k_{cat} and K_{m} for the action of pepsin on mansyl peptide substrates were determined in the manner described previously (5). Plots of v against v/S were satisfactorily linear, and indicated adherence to Michaelis-Menten kinetics.

**Fluorescence Measurements**—All measurements were made with an MPF-3 Hitachi-Perkin-Elmer fluorescence spectrophotometer in its ratio mode. The emission spectra reported here are derived from direct recorder tracings of complete spectra (~400 to 600 nm) and have not been corrected for the variation with wave length in the sensitivity of the detection system. In all experiments, the emission spectrum of a 1.9 μM solution of quinine sulfate in 0.1 N H₂SO₄ (excitation at 360 nm; uncorrected emission maximum at 448 nm) was used as the standard, and its fluorescence intensity at the emission maximum was arbitrarily set at 1.0 unit. The relative fluorescence intensity in separate experiments was normalized with this standard for fluctuations in the response of the instrument to the fluorescence of this compound; the absolute quantum yield of quinine sulfate in 0.1 N H₂SO₄ was assumed to be 0.54 (8). The uncorrected excitation maxima for the mansyl (emission near 450 nm) and dansyl (emission near 540 nm) compounds were near 335 nm and 324 nm, respectively, and these were the excitation wave lengths used. All experiments were conducted at 25°C with 1-cm cells, and the cell compartment was maintained at this temperature by means of a thermostatic jacket. Except where otherwise noted, the fluorescence spectra were scanned within 30 s of mixing the solutions of the mansyl or dansyl compound with pepsin. Separate experiments showed that, under the conditions of our studies, there was no detectable cleavage of the peptides during the first minute of incubation, except in the case of Mns-Gly-Gly-Phe-OP₄P.

**Other Materials**—The Phe-Phe derivatives used in this work (other than the mansyl compounds) were preparations the synthesis of which has been described previously (1, 2, 5). The pepstatin preparation was generously provided by Professor H. Umezawa, Institute of Microbial Chemistry, Tokyo, to whom we express our gratitude. A solution of pepstatin in methanol was used to give a 2% (v/v) concentration of methanol in the test solution. Control experiments in the absence of pepstatin were routinely run in the presence of 2% methanol.

**RESULTS**

Previous studies (1) had shown that near pH 3 the fluorescence intensity of ~10 μM dansylamide is not enhanced by the addition of an equimolar amount of pepsin, and the position of the emission maximum is not shifted to a significant extent. In the present work, most of the experiments were conducted at pH 2.35, because of the limited solubility of the mansyl compounds at higher pH values. To compare the fluorescence behavior of analogous dansyl and mansyl compounds in their interaction with pepsin, some of the earlier experiments with dansyl compounds were repeated at pH 2.35. It was found that the fluorescence of the dansyl group is partially quenched in going from pH 3.1 to pH 2.35, in agreement with the studies of Lagunoff and Ottolenghi (9) on the pH dependence of the fluorescence of the dansyl group. At the lower pH value, the emission maxima of Dns-NH₂, Dns-Phe-OP₄P, and Dns-Gly-Pro-Phe-OP₄P were in the range 540 to 550 nm, with a maximal fluorescence intensity (for a 10 μM solution) of 0.03 to 0.05 unit (normalized against quinine sulfate). Upon the addition of equimolar pepsin, there was no significant change in the fluorescence behavior of any of these dansyl compounds. In contrast to this behavior, the addition of pepsin to aqueous solutions of Mns-NH₂, Mns-Phe-OP₄P, or Mns-Gly-Gly-Phe-OP₄P produced a significant enhancement in the fluorescence of the mansyl group. For example, the maximal fluorescence intensity of 10 μM Mns-NH₂ (0.03 at 450 nm) was increased about 10-fold (0.30 at 440 nm) in the presence of equimolar pepsin; the corresponding increases for Mns-Phe-OP₄P and Mns-Gly-Gly-Phe-OP₄P were 27-fold and 37-fold, respectively. It should be added that a preparation of pepsin obtained by activation of pepsinogen and purified in the manner described by Rajagopalan et al. (10) gave the same enhancements of the fluorescence of the three mansyl compounds as did the sample of commercial crystalline pepsin used in this work.

This difference between dansyl and mansyl compounds in their apparent interaction with pepsin is a consequence, in large part, of the greater sensitivity of the mansyl group to changes in the polarity of the solvent. In Table III are given data on the effect of dielectric constant on the fluorescence of Dns-Phe-OP₄P and Mns-Phe-OP₄P; these data supplement those of Cory et al. (3) showing that the fluorescence emission of mansylamide is markedly enhanced upon decreasing the water content.
of 2-propanol-water mixtures. In relation to experiments reported later in this paper, requiring the addition of small amounts of methanol (2%, v/v), it should be noted that, in the absence of pepsin, such a change in solvent composition did not alter measurably either the emission maximum or the fluorescence intensity at that wave length for Mns-NH₃, Mns-Phe-Phe-OP₄P, or Mns-Gly-Phe-Phe-OP₄P under the conditions of these studies.

An attempt was made to determine the stoichiometry of the binding of Mns-NH₃ or Mns-Phe-Phe-OP₄P to pepsin by measurement of the enhancement of the fluorescence of these ligands at various concentrations, but because of the limited solubility of the mansyl compounds under the conditions of our studies, it was not possible to reach saturation levels. From experiments in which the pepsin concentration was varied between 3 and 71 μM and the concentration of mansyl compound was kept constant at 10 μM, estimates were made as described previously for dansyl compounds (1) of the dissociation constant (Kₛ) of the pepsin-ligand complex. Because of the low fluorescence of the unbound mansyl compounds in aqueous solution, the fluorescence intensity of the protein-ligand complex at its emission maximum (within 30 s of mixing) could be used for these calculations. These estimates of Kₛ were based on the assumption that pepsin has a single site that interacts more strongly than do other sites with each of the mansyl compounds. As will be noted later in this paper, there appear to be at least two potential binding sites for the mansyl group in pepsin, so that the calculated values of Kₛ must be considered solely as indicating the relative affinities of pepsin for the mansyl group of the compounds tested. These values (all at pH 2.35 and 25°C) were 0.2 mM for Mns-NH₃, 0.07 mM for Mns-Phe-Phe-OP₄P, and 0.03 mM for Mns-Gly-Phe-Phe-OP₄P. A value of Kₛ for the interaction of pepsin with Mns-Gly-Gly-Phe-Phe-OP₄P could not be estimated because of hydrolysis of this substrate during the time required for steady state fluorescence measurements. The value of Kₛ for Mns-Phe-Phe-OP₄P may be compared to that for Dns-Phe-Phe-OP₄P (0.13 mM at pH 3.1 and 25°C) reported previously (1), suggesting that the mansyl compound is bound somewhat more strongly.

The data in Table IV on the kinetics of the cleavage of Mns-Phe-Phe-OP₄P, Mns-Gly-Phe-Phe-OP₄P, and Mns-Gly-Gly-Phe-Phe-OP₄P indicate that the estimated Kₛ values for the first two substrates are similar to the Kₛ values obtained from fluorescence measurements. Previous work has shown (1) that the temperature dependence of Kₛ over the range 25-37°C for the hydrolysis of dansyl peptides by pepsin is negligible, the only significant effect being on kₑᵃᵗ. It will be noted in Table IV that the Kₛ values for the hydrolysis of the Phe-Phe bond of the three mansyl compounds were about the same (0.03 to 0.13 mM), and the fact that they are similar to the Kₛ values obtained from fluorescence measurements suggests that, in the action of pepsin on the mansyl peptides, Kₛ approximates Kₛ. On the other hand, the values of kₑᵃᵗ differed by as much as 8000-fold. In this respect, the action of pepsin on the mansyl compounds is comparable to that on the corresponding peptides with amino-terminal benzoyloxycarbonyl or dansyl groups; earlier work (1, 2) had shown that the enzymic cleavage of the Phe-Phe bond of a large variety of substrates of the type A-Phe-Phe-OP₄P is accompanied by relatively small changes in Kₛ and very large changes in kₑᵃᵗ, depending on the structure of the A group. Moreover, binding studies by gel filtration (11) have indicated that the major contribution to the binding energy in the formation of the enzyme-substrate complex is derived from the interaction of the Phe-Phe unit with pepsin.

In further studies on the interaction of the mansyl compounds with pepsin, advantage was taken of the discovery by Umezawa et al. (12) of a naturally occurring bacterial peptide (pepstatin) that is a powerful inhibitor of pepsin, with an estimated Kₛ of about 10⁻⁵ M near pH 2. It will be seen in Table V that the addition of equimolar pepstatin to a pepsin-mansylamide mixture did not affect markedly the enhancement of fluorescence. On the other hand, the much larger enhancement of fluorescence observed upon the addition of equimolar pepstatin to Mns-Phe-Phe-OP₄P or Mns-Gly-Phe-Phe-OP₄P was reduced by equimolar pepstatin to a value similar to that observed with Mns-NH₃. Control experiments showed that pepstatin itself did not cause a change in the fluorescence of any of the mansyl compounds under the conditions of the studies. As will be seen in Table V, the addition of 2% methanol to the mixture of pepsin and mansylamide caused a slight quenching of the fluorescence. It may be added that an increase in pepstatin concentration to 20 μM gave the same results as those obtained at the lower level.
of inhibitor. Since pepstatin forms a tight 1:1 complex with pepsin, it may be inferred that the inhibitor had blocked the active site of the enzyme, thus preventing access of the Phe-Phe unit. A less dramatic reduction of the fluorescence of pepsin-mansyl peptide mixtures is seen with the resistant synthetic substrate Z-Gly-Pro-Phe-OP4P at a level of 0.2 mM, a concentration near the \(K_m\) value (0.14 mM at pH 3.5 and 3.7) of this substrate (2). The considerable reduction in the fluorescence of equimolar mixtures of pepsin and Mns-Phe-Phe-OP4P (or Mns-Gly-Phe-Phe-OP4P) upon the addition of Z-Gly-Pro-Phe-OP4P supports the conclusion that the latter peptide also competes with the mansyl peptides for interaction at the active site of pepsin. As in the case of pepstatin, however, Z-Gly-Pro-Phe-OP4P does not affect markedly the fluorescence of an equimolar mixture of pepsin with Mns-NH2. It should be added that 0.2 mM Z-Phe-OP4P, which has an estimated \(K_m\) value of about 0.7 mM at pH 2 (5), has a negligible effect on the fluorescence of Mns-NH2 in the presence of equimolar pepsin.

It will be noted in Table V that pepsin which has been inactivated at pH 7.8 ("alkali-inactivated pepsin") exhibits a greatly reduced capacity to enhance the fluorescence of Mns-Phe-Phe-OP4P or Mns-Gly-Phe-Phe-OP4P, as compared with active pepsin. It may be surmised that in the alkali inactivated pepsin the site of interaction with the Phe-Phe unit of substrates has been modified extensively, but has not been completely abolished. On the other hand, the enhancement of the fluorescence of Mns-NH2 by the inactive pepsin was approximately the same as that observed with active pepsin. The effect of the addition of equimolar pepstatin to a 1:1 mixture of alkali-inactivated pepsin and a mansyl compound could not be tested under the conditions of these studies, because of the appearance of turbidity when pepstatin was added to inactive pepsin. In contrast to the behavior of alkali-inactivated pepsin, a solution of pepsin containing 6 M urea or 6 M guanidinium chloride caused no enhancement of the fluorescence of either Mns-NH2 or Mns-Phe-Phe-OP4P as compared with the fluorescence of these compounds in the presence of the denaturants alone. It may be concluded, therefore, that denaturation by urea or guanidinium chloride abolishes the sites in pepsin that react with the mansyl compounds. It may be added that Cory et al. (3) found the fluorescence intensity of mansyl-bovine serum albumin to be markedly reduced in the presence of 5 M guanidinium chloride.

These results suggest, as a working hypothesis, that pepsin contains at least two potential loci for binding a mansyl group. One of these appears to be associated with the active site; while it does not exhibit intrinsic affinity for the mansyl group of Mns-NH2, this locus becomes evident when the mansyl group is attached to a peptide having a Phe-Phe unit. The other locus appears to be relatively distinct from the active site, and can bind Mns-NH2 as well as the mansyl group of peptides when these are excluded from the active site by a powerful inhibitor such as pepstatin.

To test this hypothesis, the fluorescence behavior of Mns-NH2, Mns-Phe-Phe-OP4P, and Mns-Gly-Phe-Phe-OP4P was examined in the presence of equimolar pepsin that had been stoichiometrically inactivated by means of tosyl-t-phenylalanyl-diazomethane. It was known from earlier work (6) that this reagent specifically attacks the catalytic site of pepsin, and causes complete inactivation when 1 tosyl-t-phenylalanyl-methyl group (TPDM-) has been introduced per molecule of pepsin. Moreover, binding studies by the gel filtration method (13) had shown that Phe-Phe peptides are bound much less strongly by TPDM-pepsin than by untreated pepsin (11). It will be seen in Table V that although the enhancement of the fluorescence of Mns-Phe-Phe-OP4P and Mns-Gly-Phe-Phe-OP4P upon interaction with equimolar TPDM-pepsin is less than with untreated pepsin, the observed fluorescence of mansylamide is approximately 2 times greater than that found for an equimolar mixture of this compound and untreated pepsin. Moreover, the addition of pepstatin does not alter the fluorescence of mixtures of TPDM-pepsin and mansylamide or Mns-Phe-Phe-OP4P, as compared with the appropriate control, and only lowers slightly the value for Mns-Gly-Phe-Phe-OP4P.

These findings with TPDM-pepsin suggest that the introduction of the tosyl-t-phenylalanyl-methyl group at the active site of the enzyme had not only blocked that site but had also altered the conformation of the protein in such a manner as to increase the fluorescence of Mns-NH2. That this enhancement of the fluorescence of Mns-NH2 in the presence of TPDM-pepsin, as compared with that found with untreated pepsin, is not a consequence of tighter binding is indicated by the estimated value of 0.7 nm for \(K_a\) for the complex formed from TPDM-pepsin and mansylamide (pH 2.35, 25°). This value was determined in the manner described above for the estimation of the \(K_a\) values of the complexes formed from untreated pepsin and the mansyl compounds. It would appear therefore that, in the disoketone-modified pepsin, the protein conformation had been altered so as to decrease the polarity of the binding site for mansylamide to an extent sufficient to overcome the reduced binding affinity. At present it is not possible to decide whether only the presumed Mns-NH2-binding site of active pepsin had been modified as a consequence of substitution at the catalytic site, or whether one or more additional loci for binding Mns NH2 had appeared in the modified protein. In either case, it would seem that much of the pepstatin-insensitive binding of the mansyl group of Mns-Phe-Phe-OP4P or Mns-Gly-Phe-Phe-OP4P by TPDM-pepsin
occurs at a locus (or loci) which accepts Men-NH₂ and which is distinct from the blocked active site. It is noteworthy that the

\[ K_p \] values for the interaction of TPDM-pepsin with Mns-Phe-Phe-OP4P and Mns-Gly-Phe-Phe-OP4P (0.10 mM and 0.02 mM, respectively) are similar to those reported above for untreated pepsin. An interpretation of this result is made difficult by the probable multiplicity of binding loci for these compounds in TPDM-pepsin.

Another chemical modification of pepsin which, far from leading to enzyme inactivation, results in the enhancement of the rate of hydrolysis of synthetic substrates is acetylation of tyrosyl groups by acetylimidazole (14). Studies in this laboratory (7) have shown that acetyl-pepsin (containing on the average 4 or 5 acetyl groups per molecule) exhibits a markedly increased \( k_{cat} \) for the hydrolysis of the Phe-Phe bond of Z-His-Phe-Phe-OEt. It was of interest, therefore, to examine the rate of hydrolysis of synthetic substrates is acetylation of TPDM-pepsin.

The interaction of Mns-Phe-Phe-OP4P (or Mns-Gly-Phe-Phe-OP4P) with acetyl-pepsin was accompanied by a greater enhancement of the fluorescence of the mnsyl group than that observed with untreated pepsin. When equimolar peptatin was added, however, the fluorescence of Mns-NH₂ was greatly increased, suggesting that acetylation of the protein had altered the active site in such a manner that its interaction with the inhibitor produced a significant change in the conformation of the protein.

The interaction of Mns-Phe-Phe-OP4P (or Mns-Gly-Phe-Phe-OP4P) with acetyl-pepsin was accompanied by a greater enhancement of the fluorescence of the mnsyl group than that observed with untreated pepsin (Table V); the addition of equimolar peptatin produced little change in fluorescence intensity, as compared with the appropriate control values. It would seem that in acetyl-pepsin the mnsyl-binding locus associated with the interaction of a Phe-Phe unit at the active site is intact, and that the other locus, which can presumably bind the mnsyl group of Mns-NH₂ and the two peptides, has become responsive to enzyme-substrate or enzyme-inhibitor interactions at the active site. Further work is needed to test this inference, in particular to determine the \( K_i \) for peptatin in its interaction with acetyl-pepsin under the conditions of these studies. It should be added that a preparation of peptatin that had been treated in the same manner as that employed in the acetylation reaction, except for the omission of acetylimidazole from the reaction mixture, gave fluorescence data with Mns-NH₂ and Mns-Phe-Phe-OP4P similar to those obtained with untreated pepsin.

It was noted above that the mnsyl group is more sensitive than the dansyl group as a fluorescent probe for changes in the polarity of their environment, and that at pH 2.35 no significant enhancement of the fluorescence of the dansyl group of Dns-Phe-Phe-OP4P or Dns-Gly-Pro-Phe-Phe-OP4P was observed when an equimolar amount of peptatin was added. At pH 3.1, however, such effects are observed with these peptides, although not with Dns-NH₂. Thus, with 10 \( \mu \)M Dns-Gly-Pro-Phe-Phe-OP4P (emission maximum 550 nm, relative fluorescence intensity 0.07 unit), the addition of 20 \( \mu \)M peptatin shifted the maximum to 515 nm and increased the fluorescence intensity to 0.22 unit; the further addition of 20 \( \mu \)M peptatin completely reversed the effect of peptatin and shifted the emission spectrum to that of the dansyl peptide in the absence of the enzyme. The result with acetyl-pepsin was similar, except that the addition of peptatin only partly reversed the fluorescence enhancement, the resulting emission spectrum having its maximum at 535 nm (relative fluorescence intensity 0.10 unit). This suggests that peptatin is bound to acetyl-pepsin less strongly than to untreated pepsin under the conditions of these studies.

Further evidence in support of the hypothesis that peptatin has at least two binding sites for the mnsyl group, one detected by the enhancement of the fluorescence of Mns-NH₂ and the other only made evident when the NH₂-terminal mnsyl group of a peptide is "dragged" into a hydrophobic region because of the interaction of a Phe-Phe unit with the active site, is provided by fluorescence studies with peptatinogen. As shown in Fig. 1, when the fluorescence of Mns-NH₂ in the presence of peptatinogen (at pH 2.35) is followed with time, the fluorescence steadily decreases. Similar behavior was noted by Wang and Edelman (15) for the fluorescence of 2-p-toluidinylnapthalene 6-sulfonate in the presence of peptatinogen undergoing autocatalytic conversion to pepsin. They provided evidence for the view that TNS is bound by peptatinogen and peptatin at a locus distinct from the catalytic site of the enzyme. On the other hand, as will be seen from Fig. 1, the fluorescence of Mns-Phe-Phe-OP4P increases markedly upon the conversion of peptatinogen to pepsin. A similar result was reported previously (1) for the fluorescence of Dns-Gly-Pro-Phe-Phe-OP4P at pH 3.1. It is noteworthy that the enhancement of the fluorescence of Mns-Phe-Phe-OP4P and the reduction of the fluorescence of Mns-NH₂ during the activation process are both abolished by equimolar peptatin (Fig. 1). It would appear, therefore, that the conformational changes accompanying the autocatalytic conversion of peptatinogen to pepsin lead to an alteration in the binding site for mnsylamide that is distinct from the alteration in the locus for interaction with a mnsyl group that is drawn in by a Phe-Phe unit of a pepsin substrate.

**DISCUSSION**

The results in this communication confirm and extend the reports of Cory et al. (3) and of Turner and Brand (16) on the
considerable sensitivity of the mansyl group as a fluorescent hydrophobic probe. The principal disadvantage of this group, as compared with the dansyl group, is the fact that in aqueous solution the comparable mansyl compounds are more sparingly soluble. With pepsin, the greater sensitivity of the mansyl group has permitted the detection of a binding locus for mansylamide that could not be observed, under the conditions of these studies, with dansylamide. The data presented above suggest that this locus may be the one detected by Wang and Edelman (15) with TNS; as with this probe, the enhancement of the fluorescence of mansylamide decreases upon the autocatalytic conversion of pepsinogen to pepsin. We had hoped to use the acid chloride of TNS for the preparation of the corresponding amide and of N-substituted peptides, but difficulties were encountered, presumably because of the presence of a proton on the nitrogen of TNS (3). Like the mansyl compounds, TNS is only slightly fluorescent in water but fluoresces strongly when adsorbed to proteins such as bovine serum albumin or when dissolved in solvents of low polarity (3, 16).

The studies described in this paper are consistent with the hypothesis offered earlier (1) that in the interaction of pepsin with a peptide substrate of the type A-Phe-Phe-B, where the Phe-Phe bond is the only one cleaved by the enzyme, the primary interaction of the Phe-Phe unit with the catalytic region of pepsin is accompanied by important secondary interactions involving the A group. Moreover, when the A group bears an aminoterminus fluorescent probe such as the mansyl group, this group is "dragged" into an environment of lower polarity than that of the aqueous medium. It appears likely that the interaction of the Phe-Phe unit at the catalytic site facilitates the entry of the mansyl group into a region of low polarity not evident in the enzyme alone, and not accessible to the mansyl group of mansylamide. The cooperative binding resulting from such primary and secondary interactions is expressed in marked changes in the values of $k_{ma}$ for the hydrolysis of the Phe-Phe bond. Since the total binding energy (as reflected in $K_a$) in the enzyme-substrate interaction is less subject to variation as a consequence of secondary interactions, it may be surmised that the marked differences in $k_{ma}$ arise from conformational changes in the susceptible peptide or in the catalytic residues of pepsin, or in both (17).

There can be little doubt that pepsin possesses an extended active site, as might be expected of an enzyme that acts as an endopeptidase on long peptide chains. Similar extended sites have been invoked for other proteinases, for example papain (18) and elastase (19), and efforts have been made to "map" such regions in terms of discrete "subsites." As noted previously (2, 20), such "mapping" involves the assumption that these loci of interaction are associated with a relatively rigid structure in the substrate-binding area of the enzyme in question. In the case of pepsin, and possibly of other enzymes acting on oligomeric substrates, this assumption must be questioned. The large variation of $k_{ma}$ (but not of $K_a$) for a series of substrates in which a hydrophobic group X (benzoyloxy carbonyl, dansyl, mansyl) is present in substrates of the type X-Phe-Phe-Y, X-Gly-Phe-Phe-Y, X-Gly-Gly-Phe-Phe-Y, etc., together with the fluorescence data on the corresponding dansyl and mansyl compounds, make it unlikely that the extended substrate-binding site of pepsin has a discrete subsite for the X group. A more reasonable possibility is that pepsin has an extended cleft lined by hydrophobic amino acid residues, and that this cleft is not a rigid structure but is capable of conformational change during the process of enzyme-substrate interaction. On the assumption that such a cleft exists, we are attempting to estimate its extent by synthesizing higher members of the series X-(Gly)$_n$-Phe-Phe-Y ($n = 3, 4, 5$), where X is a fluorescent probe group or a benzoyloxy carbonyl group.

Although we have not yet introduced fluorescent probe groups at the COOH termini of suitable pepsin substrates, previous data (21) have indicated similar evidence of secondary interactions for substrates of the type A-Phe(NO$_2$)-Phe-B, where the A group was invariant (e.g. Phe-Gly-His). Indeed, the use of such substrates has indicated that the apparent specificity differences among several of the acid proteinases (rennin, cathespin D, mold proteinases) may depend in part on differences in such secondary interactions involving the B group (22).

The view that pepsin has an extended site lined by hydrophobic amino acid residues is supported by the strong inhibition caused by stoichiometric amounts of the isovaleryl pentapeptide pepstatin. It was noted above that the addition of equimolar pepstatin to an equimolar mixture of pepsin and Mans-Phe-OOP or Mns-Gly-Phe-Phe-OOP reduces the marked enhancement of the mansyl fluorescence to the level found for a comparable mixture of pepsin and mansylamide. It seems justifiable to infer from this result that most of the fluorescence enhancement observed with the mansyl peptides was a consequence of the entry of the mansyl group into the extended active site of pepsin, and that the mansyl group was excluded from this site by pepstatin. The relatively resistant tetrapeptide substrate Z-Gly-Phe-Phe-OP$_4$P, which is bound by pepsin much less strongly than is pepstatin, also appears to compete with the mansyl peptides for entry into the active site, as judged by the marked reduction in the fluorescence intensity.

In these studies, we have examined the effect of three kinds of inactivation on the ability of pepsin to enhance the fluorescence intensity of mansyl compounds. Denaturation by 6 M urea or guanidinium chloride abolished the enhancement, as might be expected from complete disruption of the binding sites. Inactivation of pepsin at pH 7.8 reduced the fluorescence enhancement for the two mansyl peptides, but not for mansylamide, suggesting that significant structural features of the extended active site were still intact and that the discrete binding locus for mansylamide had not been altered greatly. On the other hand, stoichiometric inactivation of pepsin by the introduction of a tosyl-L-phenylalanylmethyl group at the active site not only markedly reduced the fluorescence enhancement for Mns-Phe-Phe-OOP and Mns-Gly-Phe-Phe-OOP, but also increased the enhancement of the fluorescence of Mns-NH$_2$. Moreover, the estimated $K_a$ for the complex of mansylamide with TPDM-pepsin is about 3.5 greater than with untreated pepsin. It would appear, therefore, that in addition to blocking the catalytic site of pepsin the substitution reaction had caused a significant alteration in the configuration of the protein at the binding locus for mansylamide. This inference may be relevant to the conclusions drawn from the use of inhibited crystalline proteinases for X-ray diffraction studies, as with tosyl-chymotrypsin (23).

The finding that the enhancement of the fluorescence of mansylamide by acetyl-pepsin is similar to that of untreated pepsin, but that the addition of equimolar pepstatin greatly increases the fluorescence intensity of a mixture of acetyl-pepsin and mansylamide, suggests that upon acetylation the pepsin molecule may become more flexible. It may be that acetylation of some of the tyrosyl residues disrupts carboxylate-phenol hydrogen bonds that are important in maintaining the three dimensional structure of the protein. This apparent increase
in the possibility of conformational change observed with acetyl-
pepsin as compared with untreated pepsin may explain the large
increase in the fluorescence enhancement of the mansyl groups of
Mns-Phe-Phe-OP4P and Mns-Gly-Phe-Phe-OP4P. It is
tempting to speculate that the presumed hydrophobic cleft at
the active site of pepsin has been enlarged by partial acetylation
of the protein, thus providing the mansyl group of the mansyl
peptide with a medium of lower polarity than the one it en-
counters when dragged into the active site of untreated pepsin.
However, it is also possible that, like pepstatin, the mansyl pep-
tides interact with acetyl-pepsin at the active site to alter the
conformation at the mansylanlaide-binding locus so as to provide
an environment of lower polarity for the mansyl group of peptide
molecules not bound at the active site. A decision on these
possibilities cannot be made on the basis of the available infor-
mation, but the fact that acetyl-pepsin hydrolyzes synthetic
substrates with a higher $k_{cat}$ and unchanged $K_m$ (7), together
with the fluorescence data presented above, argues for con-
siderable conformational flexibility in the catalytically active
enzyme.

It should be recalled that Wang and Edelman (15) showed a
peptide released upon the activation of pepsinogen to be a pepsin
inhibitor and to enhance the fluorescence of TNS bound to
pepsin. They suggested that this fluorescence enhancement may be a
reflection of conformational changes at a discrete TNS-
binding site, as a consequence of the interaction of the inhibitor
at the active site. Because of the close similarity in
structure of the chromophores of TNS and mansylanlaide, it may
be surmised that the latter also binds at the presumed TNS-
binding site. The results reported in this communication sup-
port the suggestion of Wang and Edelman (15) and further
indicate the possibility of considerable conformational flexibility
at the active site of pepsin in response to enzyme-substrate (or
enzyme-inhibitor) interaction. The finding that mansylanlaide
does not appear to interact with the protein at the active site,
but that the mansyl group of suitable mansyl peptides is dragged
into that site, raises the possibility that a substrate binding
cleft in pepsin is formed through conformational changes arising
from the interaction of a suitable peptide substrate or inhibitor
with active site groups of the enzyme. Such a cleft may be
present in pepsinogen, but occupied by the amino-terminal
portion that is removed by proteolysis during the activation
process; upon conversion to pepsin, the cleft may be narrowed
after the release of the activation peptides. This narrowed
cleft may be inaccessible to the mansyl group of mansylanlaide,
but may be widened by the specific interaction of a suitable
peptide substrate with the catalytic site of the enzyme. Studies
on optical rotation and circular dichroism have indicated that
significant conformational changes occur during the conversion
of pepsinogen to pepsin, and as a consequence of the interaction
of pepsin with a peptide substrate (24).

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