Fluorescence technique for comparative studies of substrate-binding subsites in serine proteinases

Application to subtilisins

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A fluorescence technique for comparative studies of substrate-binding subsites in serine proteinases is described. It consists of: (1) selective labelling of the corresponding subsites with a fluorescent group by using *N* α -dansyl(5-dimethylaminonaphthalene-1-sulphonyl)ated peptide chloromethanes containing different numbers of amino acid residues, and (2) probing the immediate environment of the subsites by quenching experiments using ionic and neutral quenchers. Intramolecular distances between the subsites and particular chromophores can be also determined. The technique is of general applicability to all serine proteinases. The abovementioned approach was applied to two proteinases: subtilisin Novo and mesentericopeptidase. It was concluded that the substrate-binding site of mesentericopeptidase is considerably more polar than that of subtilisin Novo. Intramolecular distances between the labelled subsites and tryptophan residues in the two proteinases were determined.

INTRODUCTION

The strategies often employed in investigating substrate-binding sites in serine proteinases are kinetic measurements and X-ray-crystallographic studies on enzyme-peptide-inhibitor conjugates. We have used the following procedures for comparative studies of binding subsites in the two serine proteinases subtilisin Novo and mesentericopeptidase.

(1) Specific labelling of the subsites with a highly fluorescent group using a series of *N* α -dansylated peptide chloromethanes containing different numbers of amino acid residues.

In this way the fluorescent dansyl moiety will occupy the subsite next to that engaged with the *N*-terminal amino acid residue. Thus the dansyl group of the inhibitor Dns-Ala-Phe-CH₂Cl will bind to subsite S₃ [nomenclature of Schechter & Berger (1967)] etc.

(2) Probing the immediate environment of the corresponding subsites, containing a bound fluorescent group, by quenching experiments using ionic and neutral quenchers.

This provides information about the polarity of the fluorophore environment and the degree of its exposure to the solvent. This probing technique can be also applied to study conformation changes in the region of substrate-binding subsites. Intramolecular distances between the selectively labelled subsites and particular chromophores in the proteinase of interest can be measured by energy-transfer studies.

The advantage of the method described here is determined mainly of its high sensitivity and selectivity. It is of general applicability to all serine proteinases. We believe that this approach will be especially useful in

studies of substrate-binding sites of serine proteinases for which X-ray-crystallographic data are not available.

In the present paper we discuss the results of its application to two subtilisin-type serine proteinases: subtilisin Novo and mesentericopeptidase.

MATERIALS AND METHODS

Subtilisin Novo was generously given by Professor Ib Svendsen (Department of Chemistry, Carlsberg Laboratory, Copenhagen, Denmark). Mesentericopeptidase was isolated in a homogeneous state as described by Karadjova *et al.* (1970). The peptide chloromethanes Z-Ala-Phe-CH₂Cl, Z-Ala₂-Phe-CH₂Cl and Z-Ala₃-Phe-CH₂Cl were kindly supplied by Dozent Dr. S. Fittkau (Institute of Biochemistry, Halle-Wittenberg, German Democratic Republic). They were converted into the corresponding *N* α -dansylated derivatives as described by Peters *et al.* (1984), with slight modifications. The enzyme-peptide-inhibitor conjugates were purified by gel chromatography and the introduction of the dansyl group was confirmed spectrophotometrically.

Dansyl chloride was purchased from BDH Chemicals (Poole, Dorset, U.K.). Dansyl fluoride was synthesized from dansyl chloride by the method of Vaz & Schoellmann (1976a). Dansyl-subtilisins were prepared as described for subtilisin DY by Genov *et al.* (1982).

Fluorescence spectra were recorded with a Perkin-Elmer model LS 5 spectrofluorimeter equipped with a Data Station model 3600. The absorbance of the samples was lower than 0.05 at $\lambda_{\text{excitation}} = 295 \text{ nm}$ to avoid inner-filter effects. All measurements were performed at pH 7.0 and at 20 °C. A quantum yield of 0.147 for the

Abbreviations used: Dns (in chloromethane structures) or dansyl, 5-dimethylaminonaphthalene-1-sulphonyl; Z, benzyloxycarbonyl; -CH₂Cl, chloromethane; H₄furan, tetrahydrofuran.

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tryptophan solution in water was used (Parker & Rees, 1960).

Fluorescence quenching was analysed by using the Stern–Volmer equation (Weller, 1961):

$$F_0/F = 1 + K_{SV}[q]$$

where F_0 and F are the fluorescence intensities at an appropriate wavelength in the absence and presence of quencher respectively, K_{SV} is the collisional quenching constant and q is the quencher. The inner-filter effect due to acrylamide was corrected by the factor:

$$Y = \text{antilog} (d_A + d_E)/2$$

where d_A and d_E are the absorbances at the excitation and emission wavelengths respectively.

Electronic excitation energy transfer between the tryptophan residues and the uniquely subtilisin-bound dansyl group was estimated according to Förster's (1948) Theory by using the equation:

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

where r is the distance between the energy donor and acceptor, E is the energy-transfer efficiency and can be determined as follows:

$$E = 1 - Q_{D-A}/Q_D$$

where Q_{D-A} and Q_D are the donor quantum yields in the presence and in the absence of an acceptor respectively.

R_0 , the distance for a 50% probability of energy transfer is:

$$R_0 = (J_{AD} \cdot K^2 \cdot Q \cdot n^{-4})^{1/6} (9.79 \times 10^3) \text{ nm}$$

where Q is the quantum yield of the donor in the absence of acceptor.

$$J_{AD} = \int_0^\infty F_D(\nu) \cdot \epsilon_A(\nu) \cdot \nu^{-4} \cdot d\nu$$

is the overlap integral between $\epsilon_A(\nu)$, the molar absorptivity of the acceptor, and $F_D(\nu)$, the donor emission spectrum on a wavenumber scale, normalized to unity. n is the refractive index of the medium. K^2 is the orientation factor determined by the orientation in the space of the transition dipole moments of the donor and acceptor.

Absorption spectra were obtained with a Shimadzu model 3000 spectrophotometer.

The caseinolytic activity was determined by the method of Kunitz (1935).

RESULTS AND DISCUSSION

The dansylated peptide chloromethanes when allowed to react with subtilisin Novo and mesentericopeptidase produced full loss of proteolytic activity. We have employed inhibitors (Dns-Ala-Phe-CH₂Cl, Dns-Ala₂-Phe-CH₂Cl and Dns-Ala₃-Phe-CH₂Cl) in which the amino acid residues in positions P₃, P₄ and P₅ were replaced by a dansyl group (nomenclature of Schechter & Berger, 1967). The peptide inhibitors were designed to label specifically with a fluorescent group the subsites S₃, S₄ and S₅. Fluorescent properties of the model compound dansylalanine were also studied.

It was shown by crystallographic studies that the mode of binding of peptide chloromethanes is a good model for substrate binding to serine proteinases and that *N*-

terminal blocking groups occupy the subsite next to that taken by the *N*-terminal residue (Robertus *et al.*, 1972). Thus, in the subtilisin–inhibitor conjugates, the fluorescent-dansyl group was placed one subsite further from the corresponding point of attachment of P₂, P₃ or P₄. In the case of subtilisins it is not possible to label subsite S₁ with dansyl fluoride because these proteinases possess two different productive substrate-binding sites: A, a site for binding peptide chloromethanes, and the so-called 'B'-site, which binds small aromatic inhibitors such as benzoylarginine, phenylmethanesulphonyl fluoride or dansyl fluoride (Wright, 1972; Vaz & Schoellmann, 1976b). Subsite S₂ in subtilisins was not probed because in this case we had to use Dns-Phe-CH₂Cl. It is known that substrates which are not peptides but modified amino acids could bind productively either in site A or in site B (Bosshard, 1973). Of course, for other serine proteinases, where there are no such limitations, the approach can be applied for studying S₁ and S₂ too. Here we used dansyl fluoride for the preparation of dansyl-subtilisins in order to investigate the B-site. For that reason fluorescence measurements on dansyl-labelled subtilisin Novo and mesentericopeptidase permitted characterization of the subsites S₃, S₄, S₅ and the B-site.

The results from the fluorescence and energy-transfer measurements on subtilisin Novo and mesentericopeptidase containing bound dansylated inhibitors are presented in Table 1. Ionic quenchers are hydrated and charged and they should be able to quench only surface chromophores. In all cases the K_{SV} values for the quenching of the bound fluorophore emission by iodide ions are higher than that for the model compound dansylalanine in water. A fourfold increase of the quenching constant was observed when the dansyl group was in the position S₅ or at the B-site of mesentericopeptidase. These findings could be explained by a positive charge of the environment. It can be concluded also that the environments of S₄, S₅ and B-site differ in subtilisin Novo and mesentericopeptidase.

There was no quenching effect of Cs⁺ ions on the fluorescence of the dansylated compounds here described.

Acrylamide is an excellent uncharged quenching probe and can quench the excited fluorophores regardless of whether they are located on the surface or in the interior of the protein globule (Eftink & Ghiron, 1976). The quenching constants obtained by this compound are six to eight times greater when the dansyl group is bound to the subsites of subtilisin Novo or to the S₃ or B-site of mesentericopeptidase in comparison with the value for dansylalanine in water (Table 1). The K_{SV} values for the dansyl group in positions S₄ and S₅ in mesentericopeptidase are 18 or 13 times lower than the values for the corresponding subsites in the Novo enzyme. Experiments with dansylalanine in water or dioxan and H₂furan showed that the intensity of the dansyl emission and the quenching constants with acrylamide increase significantly in apolar media. This could be associated with a quenching effect of water molecules on the excited dansyl group. The results showed that the substrate-binding site of subtilisin Novo is considerably less polar than that of mesentericopeptidase. The biggest difference is at S₄ and S₅. The environment's polarity of the last two subsites in mesentericopeptidase is very similar. Likewise, the polarity of the S₃ and S₄ environments in the Novo enzyme is equal.

Table 1. Fluorescence and quenching parameters for a single dansyl group specifically introduced into binding subsites in subtilisin Novo and mesentericopeptidase

Subsite at which dansyl group is located	Quenching parameters for the bound dansyl group		Emission-maximum position of the dansyl group(nm)	Energy-transfer efficiency (Trp-Dns) (<i>E</i>)	Dns-Trp distances (nm)
	<i>K</i> _{SV} (I ⁻)* (M ⁻¹)	<i>K</i> _{SV} (acrylamide) (M ⁻¹)			
Subtilisin BPN'/Novo					
S ₃ (Dns-Ala-Phe-CH ₂ Cl)	0.6	18.0	502	0.82	1.88 ± 0.1
S ₄ (Dns-Ala ₂ -Phe-CH ₂ Cl)	0.6	18.1	502	0.83	1.73 ± 0.1
S ₅ (Dns-Ala ₃ -Phe-CH ₂ Cl)	0.8	13.3	500	0.80	1.37 ± 0.1
B (dansyl fluoride)	0.6	15.9	505	0.54	1.93 ± 0.1
Mesentericopeptidase					
S ₃ (Dns-Ala-Phe-CH ₂ Cl)	0.5	11.0	505	0.20	2.65 ± 0.1
S ₄ (Dns-Ala ₂ -Phe-CH ₂ Cl)	1.0	1.1	520	0.6	1.67 ± 0.1
S ₅ (Dns-Ala ₃ -Phe-CH ₂ Cl)	1.5	1.2	520	0.64	1.50 ± 0.1
B (dansyl fluoride)	1.5	14.2	505	0.57	1.81 ± 0.1
Dansylalanine in:					
100% Water	0.4	2.4	535	—	—
H ₄ furan					
20%	—	—	534	—	—
40%	—	—	523.5	—	—
60%	—	—	518	—	—
80%	—	—	517	—	—
100%	—	—	491.5	—	—

* I⁻ is iodide ion; $K_{SV}(I^-)$ is the collisional quenching constant obtained in the presence of iodide ions.

The emission properties of the dansyl group are very informative about the polarity of its environment. Experiments with the model compound dansylalanine in H₄furan (Table 1) showed that the emission transition energies decrease with increasing solvent polarity. Evidently the environment of the subsites in the Novo and mesentericopeptidase enzymes is significantly less polar than that created by water. The emission maximum position of the dansyl group bound to S₄ or S₅ subsites in the latter proteinase differs considerably from the corresponding λ_{max} for the other subsites in the two enzymes, confirming the data from the quenching experiments with acrylamide. Our results are consistent with the crystallographic data for the substrate-binding sites in subtilisin BPN'/Novo (Wright, 1972; Kraut, 1977).

Distances between the dansyl group selectively introduced at the corresponding subsites and the tryptophan residues in the two proteinases were estimated from the singlet-singlet energy-transfer studies (Tables 1). The energy-transfer efficiency depends not only on the distance between the chromophores but also on the orientation in the space of the transition dipole moments of the donor and acceptor, the refractive index of the medium, the overlap of the absorption and fluorescence spectra and the donor fluorescence quantum yield. The estimation of the intramolecular distances is limited, mainly due to the uncertainty in the orientation factor, K^2 . There are several reasons for accepting a value of 0.476 for K^2 in the case of subtilisin Novo and mesentericopeptidase containing dansyl groups at the respective binding subsites.

(1) The tryptophan residues in subtilisin Novo and mesentericopeptidase are to some extent 'buried' (Hol, 1971; Genov & Jori, 1973). They should be involved in

intramolecular interactions, and preferred orientations of the indole rings can be assumed.

(2) We have built three-dimensional models of the complexes between the N^α-dansylated peptide chloromethanes and the active site of the two subtilisins. The models showed that the orientation of the dansyl group is essentially fixed.

It is reasonable to accept that a 'fixed' acceptor and a 'fixed' array of randomly oriented donors exist in the conjugates described in the present paper. It was shown (Maksimov & Rozman, 1961) that, in such a case, K^2 is equal to 0.476, and we used this value for the calculation of R_0 . By this means, if K^2 is set equal to 2/3, the value for random orientation, ' r ', will change only by 0.1 nm. A value of 1.36 was accepted for ' n ' (Horrocks & Collier, 1981).

The indole rings occupy the same positions (106, 113 and 241) in the two proteinases. Trp-106 is the major source of tryptophan emission (Shopova & Genov, 1983). The distances between S₄, S₅ and the B-site on the one hand and the indole groups on the other are very similar for both of the enzymes, suggesting similarities in the spatial localization of these subsites.

The values for energy-transfer efficiency for the Novo enzyme are greater than those for mesentericopeptidase. A fourfold decrease of the efficiency was observed when the dansyl group was bound to the S₃ subsite in the latter enzyme instead of to S₃ in Novo. The difference in the energy-transfer efficiency and in the distance dansyl-in-S₃-Trp observed for subtilisin Novo and mesentericopeptidase can be explained by a different contribution of the particular tryptophan residues to the total emission or with differences in the spatial location of the dansyl group in the two enzyme-inhibitor complexes.

The quenching of the indole fluorescence by the dansyl group introduced at the corresponding subsites was connected with a bathochromic shift of the emission maximum position of about 10–15 nm. Exclusively high efficiency of the energy transfer for the pairs dansyl-in-S₃-Trp, dansyl-in-S₄-Trp and dansyl-in-S₅-Trp in subtilisin Novo was observed. About 80% of the tryptophan emission was quenched in these cases. This result is consistent with the conclusion that, in subtilisin Novo, Trp-106 is the major source of indole emission, because the dansyl group in positions S₃, S₄ and S₅ should be very near to this residue according to the crystallographic model of the same enzyme. The spectrum recorded after the quenching of Trp-106 emission is probably due to Trp-241, because Trp-113 is not, or is very weakly, fluorescent (Brown *et al.*, 1977). Consequently, the environment of Trp-241 is considerably more polar than that of the other two indole rings, and this leads to the shift of the emission maximum position at 352–356 nm.

Subtilisin Novo and mesentericopeptidase are two closely related serine proteinases, and only quantitative differences in their specificity were observed (Genov & Shopova, 1974). However, considerable differences between their substrate-binding sites were revealed by using the fluorescence technique described in the present paper. This technique is applicable to all serine proteinases and permits the discovery of subtle differences among these enzymes.

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