Intramolecular distances between tryptophan residues and the active-site serine residue in alkaline bacterial proteinases as measured by fluorescence energy-transfer studies

Nicolay Ch. GENOV,*‡ Maria SHOPOVA,* Raina BOTEVA,* Fernanda RICCHELLI†
and Giulio JORI†

**Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Sofia 1040, Bulgaria, and †Institute of Animal Biology, C.N.R. Centre for Haemocyanins and Other Metalloproteins, University of Padova, Padova 35100, Italy*

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Singlet–singlet energy transfer from the tryptophan residues to an active-site-serine-bound 5-dimethylaminonaphthalene-1-sulphonyl group was investigated in four subtilisins. The transfer distances for subtilisin Novo and mesentericopeptidase are 1.93 ± 0.20 nm (19.3 ± 2.0 Å) and 1.81 ± 0.20 nm (18.1 ± 2.0 Å) respectively. The positions of the indole groups in the three-dimensional structures of the two pairs of proteinases, namely subtilisin Novo and mesentericopeptidase on the one hand and subtilisins Carlsberg and DY on the other, are essentially identical.

At present, Förster's theory of electronic energy transfer (Förster, 1948) is widely used as a 'spectroscopic ruler' for the determination of intramolecular distances in proteins (Dale & Eisinger, 1974; Stryer, 1978; Horrocks & Sudnic, 1981; Dunn *et al.*, 1981). The Dns group can be specifically bound (Vaz & Schoellmann, 1976a) to the active-site serine residue of serine proteinases and represents a suitable 'reporter group' owing to the overlap of its absorption spectrum with the tryptophan emission. The applicability of such investigations for measuring intramolecular distances in proteins was demonstrated in the cases of chymotrypsin and trypsin. A good correlation between the fluorescence and X-ray-crystallographic data for the mean separation between the selectively bound Dns group and the tryptophan residues in these enzymes has been observed (Vaz & Schoellmann, 1976a,b).

In the present paper we report energy-transfer investigations on four alkaline bacterial proteinases: mesentericopeptidase and subtilisins Novo, Carlsberg and DY. The active-site serine residue of each of these enzymes was selectively labelled with a Dns group. Subtilisins Carlsberg and DY each contain a single tryptophan residue per molecule (Trp-113) and a distance between two definite chromophores could be evaluated in this case. The

situation with the other two proteinases is more complicated because they each contain three tryptophan residues per molecule. However, in previous papers (Ricchelli *et al.*, 1981; Shopova & Genov, 1983) we have shown that one indole ring (Trp-106 in subtilisin Novo) is the major source of emission, thus facilitating the data interpretation and the comparison with the X-ray-crystallographic model of subtilisin Novo.

Materials and methods

Mesentericopeptidase and subtilisin DY were purified and isolated in a homogeneous state as described previously (Karadjova *et al.*, 1970; Genov *et al.*, 1982). Subtilisins Carlsberg and Novo were a gift from Professor Ib Svendsen (Carlsberg Laboratory, Copenhagen Valby, Denmark). L-Tryptophan was purchased from Merck (Darmstadt, Germany), and Sephadex G-75 was from Pharmacia (Uppsala, Sweden). Dns chloride was obtained from BDH Chemicals (Poole, Dorset, U.K.); Dns fluoride was synthesized from Dns chloride by the method described by Vaz & Schoellmann (1976a); Dns-subtilisins were prepared according to the procedure used for subtilisin DY (Genov *et al.*, 1982).

The molar absorption coefficients of the Dns derivatives were calculated as follows: the absorption spectra of the protein solutions at pH 7.0 were recorded and the protein concentrations were

Abbreviation used: Dns, 5-dimethylaminonaphthalene-1-sulphonyl.

‡ To whom correspondence should be addressed.

determined by amino acid analysis. A Mikrotechna (Prague, Czechoslovakia) model AAA 881 automatic amino acid analyser was used.

Fluorescence measurements were performed with Perkin-Elmer spectrofluorimeter model MPF-44 equipped with a Corrected Spectrum Accessory or model 650-40, which is microprocessor-controlled. The samples were dissolved in 0.1 M-NaCl, pH 7.0. The absorbance of the solutions was lower than 0.05 at $\lambda_{\text{exc.}} = 300\text{ nm}$ to avoid inner-filter effects. The emission quantum yields were determined by comparing the protein emission spectra with those of a tryptophan solution in water ($Q = 0.147$) (Parker & Rees, 1960; Wiget & Luisi, 1978).

Singlet-singlet energy transfer from the indole rings to the uniquely protein-bound Dns group was estimated by using the equation (Matsumoto & Hammes, 1975; Vaz & Schoellmann, 1976b):

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

where R_0 is the critical distance for a 50% probability of energy transfer, Q is the donor fluorescence quantum yield in the absence of an acceptor, n is the refractive index of the medium, K^2 is the orientation factor determined by the orientation in space of the transition dipole moments of the donor and acceptor, and

$$J_{\text{AD}} = \int_0^\infty F_{\text{D}}(v) \cdot \epsilon_{\text{A}}(v) \cdot v^{-4} \cdot dv$$

is the overlap integral between $\epsilon_{\text{A}}(v)$, the decadic molar absorptivity of the acceptor, and $F_{\text{D}}(v)$, the donor emission spectrum on a wavenumber scale, normalized to unity (Conrad & Brand, 1968). The distance r between the energy donor and acceptor was estimated by the expression:

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

where E is the energy-transfer efficiency. E can be related to the quantum yields as follows:

$$E = 1 - Q_{\text{D} \rightarrow \text{A}}/Q_{\text{D}}$$

where $Q_{\text{D} \rightarrow \text{A}}$ and Q_{D} are the donor quantum yields in the presence and in the absence of an acceptor respectively.

U.v.-absorption spectra of the various Dns derivatives were obtained with a Beckman UV 5270 spectrophotometer.

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

Results and discussion

The inactivation of the four proteinases by Dns fluoride follows first-order kinetics (Fig. 1). Table 1 summarizes absorption and emission characteristics

Fig. 1. Time-dependence of subtilisin inactivation with Dns fluoride at 20°C

Phosphate buffer (50 mM), pH 7.0, was used. For further details see the text. ●, Mesentericopeptidase; ■, subtilisin Carlsberg; ▲, subtilisin Novo; ▼, subtilisin DY.

Table 1. Absorption and emission characteristics of the Dns group selectively introduced at the active site of subtilisins

Enzyme	Absorption characteristics		
	Long-wavelength absorption maximum position of the bound Dns group (nm)	Molar absorption coefficient of the bound Dns group at the long-wavelength maximum ($\text{M}^{-1} \cdot \text{cm}^{-1}$)	Emission maximum position of the bound Dns group after excitation at the long-wavelength maximum (nm)
Dns-subtilisin Novo	345	4.7×10^3	505
Dns-mesentericopeptidase	317	4.6×10^3	505
Dns-subtilisin Carlsberg	325	4.3×10^3	507
Dns-subtilisin DY	323	4.1×10^3	502

Table 2. *Dns-tryptophan distances in subtilisins with specifically labelled active sites as measured by fluorescence energy transfer*

Enzyme	Quantum yield of the donor in the absence of an acceptor	Transfer efficiency <i>E</i>	Spectral overlap integral $J_{AD} \times 10^{15}$ ($\text{cm}^6 \cdot \text{mmol}^{-1}$)	Dns-Trp distances <i>r</i>	
				(nm)	(Å)
Dns-subtilisin Novo	0.110	0.54	4.55	1.93 ± 0.20	19.3 ± 2.0
Dns-mesentericopeptidase	0.110	0.57	3.45	1.81 ± 0.20	18.1 ± 2.0
Dns-subtilisin Carlsberg	0.022	0.35	4.80	—	—
Dns-subtilisin DY	0.019	0.37	3.20	—	—

of the modified proteins obtained after gel filtration of the reaction mixtures through a column of Sephadex G-75. The long-wavelength absorption maximum of the bound Dns group is located in the 317–345 nm region, which is indicative of differences in the microenvironment of the Dns chromophore. Similar variations were observed for different proteins by other authors (Vaz & Schoellmann, 1976a). The results for the molar absorption coefficients (ϵ_m) of the bound chromophore are in good agreement with the value of the molar absorption of the Dns group in ethyl 5-dimethylaminonaphthalene-1-sulphonate at its long-wavelength absorption maximum ($4.2 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 342 nm) (Vaz & Schoellmann, 1976a). A good correlation between the values for ϵ_m of the introduced chromophore and the degree of inactivation was observed; the interaction of about 1 mol.prop. of the reagent with 1 mol.prop. of the enzyme was accompanied by full loss of the catalytic activity. For that reason, it appears that the interaction between the reagent and each subtilisin is approximately 1:1 and leads to a selective labelling of the active site.

Subtilisin Novo and mesentericopeptidase each contain three tryptophan residues per molecule, whereas subtilisins Carlsberg and Dy each contain a single tryptophan residue per molecule (Trp-113, according to the nomenclature adopted for the Novo enzyme). Upon 300 nm-excitation native proteinases exhibit exclusively tryptophan emission. This emission is strongly quenched by the active-site-serine-bound Dns group, and the efficiency of energy transfer for the various proteinases ranges between 35% and 57% (Table 2).

Determinations of intramolecular distances by energy-transfer studies are limited by the uncertainty in the orientation factor K^2 , which can have a value between 0 and 4. The tryptophan residues in subtilisins can be expected to be involved in intramolecular interactions and hence to assume preferred orientations (Poland & Scheraga, 1965). It is known from the X-ray-crystallographic model of subtilisin Novo (Hol, 1971) that all three indole rings are to some extent 'buried', being partially blocked

by neighbouring amino acid side chains and polypeptide segments. It is also shown that the tryptophan residues in mesentericopeptidase are to some extent 'buried' (Genov & Jori, 1973), and that this proteinase and subtilisin DY possess a strong similarity to subtilisins Novo and Carlsberg respectively, particularly as regards the microenvironment of the aromatic residues (Ricchelli *et al.*, 1981; Genov *et al.*, 1982). Moreover, the Dns-binding site in subtilisin Carlsberg represents the apolar site 'B' (Vas & Schoellmann, 1976b), which binds the aromatic portion of small-molecular-mass subtilisin inhibitors. In view of the structural homology of the alkaline bacterial proteinases, such a situation exists in all four Dns-subtilisins, i.e. the orientation of the Dns group in these conjugates is essentially fixed. On the basis of these considerations it is reasonable to accept that the spatial orientation of the donor and acceptor dipoles in Dns-subtilisin Novo and Dns-mesentericopeptidase approaches closely that of a 'fixed' acceptor and a 'fixed' array of randomly oriented donors; hence we have used $K^2 = 0.476$ (Maksimov & Rozman, 1961). Each of the two proteinases contains three tryptophan chromophores per molecule, and it is reasonable to accept the presence of 'an array of randomly oriented donors'. Trp-106 in subtilisin Novo is the major (but not the only) source of indole fluorescence (Shopova & Genov, 1983). A similar situation exists in mesentericopeptidase (Ricchelli *et al.*, 1981). A value of 1.36 was accepted for n (Horrocks & Collier, 1981).

The distance of $1.93 \pm 0.20 \text{ nm}$ ($19.3 \pm 2.0 \text{ Å}$) (Table 2), determined from the energy-transfer data for Dns-subtilisin Novo, which can serve as a model, is in a very good agreement with the value of 1.18 nm (18.1 Å) calculated from the atomic coordinates (Hol, 1971) of the X-ray-crystallographic model of the same enzyme as the distance between the O-G atom of the active-site Ser-221 and the C-D2 atom of Trp-106 (Table 3). This agreement confirms the conclusions that Trp-106 is the major source of tryptophan emission in subtilisin Novo (Shopova & Genov, 1983) and suggests that in the case of the two enzymes mentioned above it is

Table 3. *Active-site-serine-tryptophan distances in subtilisin Novo according to the X-ray-crystallographic model*

We have calculated the distances from atomic co-ordinates given by Hol (1971).

Atoms	Distance	
	(nm)	(Å)
O-G atom of Ser-221 – C-D2 atom of Trp-106	1.81	18.1
O-G atom of Ser-221 – C-D2 atom of Trp-113	2.12	21.2
O-G atom of Ser-221 – C-D2 atom Trp-241	2.78	27.8

reasonable to set K^2 equal to 0.476. It can be shown that if K^2 is set equal to $\frac{2}{3}$, i.e. the value for random orientation, the estimated distance r will change with only 0.10 nm (1.0 Å). It was shown by Dale & Eisinger (1974) that in the case of the one chromophore transition moment 'fixed' and the other 'random' K^2 can only vary between $\frac{1}{3}$ and $\frac{2}{3}$. This will make for a ± 0.20 nm (± 2.0 Å) difference in the values of r . The results presented in Table 2 show that the positions of the tryptophan residues in the three-dimensional structures of subtilisin Novo and mesentericopeptidase are similar.

Having in mind the inaccuracy that could arise from the very low quantum yield of the single tryptophan residue (Trp-113) in subtilisins Carlsberg and DY, we did not calculate the Dns-tryptophan distance in these enzymes. However, the similarity of the values obtained from the quantum yields, spectral overlap integrals and transfer efficiencies (Table 2) leads to the conclusion that the corresponding distances are very similar, i.e. the positions of the single indole groups in the three-dimensional

structure of subtilisins Carlsberg and DY are essentially identical.

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