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Fluorescence properties of subtilisins and related proteinases (subtilases): relation to X-ray models

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Abstract

The fluorescence properties of six subtilases with known X-ray structure were determined using the same experimental conditions and instrumentation. The steady state and nanosecond lifetime measurements were performed on purified samples of phenylmethanesulphonyl-inhibited proteinases in the presence of 20 mM $CaCl_2$ which stabilizes the molecules. The tryptophan emission quantum yield strongly depends on the local environment and varies from 0.02 to 0.10. The efficiency of tyrosine-to-tryptophan energy transfer also varies (0%–70%) in the different enzymes; the most efficient transfer was observed for thermitase.

Experiments with nanosecond excitation indicated that the tryptophan fluorescence of subtilases decays with two exponential components. The X-ray models of the six proteinases were analysed in the region of the tryptophyl residues and were used to explain the observed properties.

Keywords: Fluorescence, Lifetime, Subtilisin, Subtilases, X-ray models

1. Introduction

Subtilisins and related proteolytic enzymes, the so-called subtilases (EC 3.4.21.14), are widespread serine proteinases found in bacteria, fungi, yeasts and higher eukaryotes [1]. They were first isolated from bacteria and it was thought that this kind of enzyme was limited to prokaryotic microorganisms. However, many new members of this protein family have been isolated during the past few years, not only from prokaryotes, but also from lower and higher eukaryotes including insects, plants and mammals [1].

Intensive studies on the structure–function relationships in subtilases have been performed during recent decades, *e.g.* the determination of amino acid sequences, three-dimensional structures, substrate specificity, organization of the active site and mechanism of catalysis [1, 2]. Subtilases are the most highly investigated biocatalysts and the large data base makes them attractive objects for the application of protein engineering [3]. They have been used as model systems for the alteration of enzyme properties, *e.g.* the catalytic function of the active site [4], substrate specificity [5, 6] and thermal stability [7, 8]. Subtilisins and related enzymes, in particular the thermostable and alkalophilic proteinases from microorganisms, continue to be of increasing interest, not only as models for understanding the mechanism of enzyme catalysis and the molecular basis of thermostability [9], but also due to their industrial importance. These enzymes are produced on an industrial scale and find application as biologically active components of washing powders.

Fluorescence spectroscopy is one of the most sensitive methods for studying protein conformation in solution and changes in conformation under the influence of various factors. Such molecular information is obtainable because the emission properties of tryptophyl and tyrosyl sidechains, which are the main intrinsic protein fluorophores, are dependent on their microenvironment. The tryptophyl fluorescence parameters are considerably more informative than those of the tyrosyl residues. The best method of interpretation of the fluorescence data is in terms of selective effects on a single or limited number of fluoro-

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phores. The complexity of the emission spectra can be limited or even avoided by selective excitation of the protein indole groups.

In this paper, we describe the fluorescence properties of five subtilases whose three dimensional structure has been determined by X-ray crystallography: alkaline mesentericopeptidase (SBMEP) from *Bacillus mesentericus* [10], subtilisin Novo (SBNOVO) from *Bacillus amyloliquefaciens* which is identical with subtilisin BPN [11, 12], subtilisin Carlsberg (SBCARL) from *Bacillus licheniformis* [13], proteinase K (SBPRK) from *Tritirachium album Limber* [14] and thermitase (SBTHER) from *Thermoactinomyces vulgaris* [15]. In addition, fluorescence data from subtilisin DY (SBDY) from the DY strain of *Bacillus subtilis* [16] are included. X-Ray studies on the structure of the last enzyme are now in progress [17]. The steady state and dynamic fluorescence parameters of the six proteinases were determined using the same experimental conditions and instrumentation, allowing the comparison of the fluorescence properties of the proteins investigated. In all experiments proteinases inactivated by phenylmethanesulphonylfluoride (PMSF) were used to avoid autolysis. Measurements were performed in the presence of 20 mM $CaCl_2$ which stabilizes the enzyme structure. Use of identical $CaCl_2$ concentrations for the different subtilisins is particularly important for a valid comparison of their properties: use of different conditions makes it difficult to compare previous results for individual enzymes. The emission spectral data are related to the respective crystallographic models and are used to characterize the environment of the fluorophores.

2. Materials and methods

SBNOVO and SBCARL were generously donated by Professor Ib Svendsen (Department of Chemistry, Carlsberg Laboratory, Copenhagen). SBPRK was purchased from Sigma Chemical Company (St. Louis). SBMEP was isolated in a homogeneous state as described by Karadjova *et al.* [18]. SBDY was purified by the method of Genov *et al.* [16]. SBTHER was generously donated by Dr. W. E. Hohne (Institute of Biochemistry, Humboldt University, Berlin). Sephadex G-75 was a product of Pharmacia (Uppsala, Sweden). All other chemicals used were of analytical grade. Phenylmethanesulphonyl (PMS) derivatives of the six proteinases were prepared by the procedure described in ref. 16.

Fluorescence measurements were performed using a Perkin–Elmer model LS 5 spectrofluorometer equipped with a thermostatically controlled assembly and a Data Station model 3600. The optical absorbance of the solutions was lower than 0.05 at the excitation wavelength to avoid inner filter effects. Excitation at 300 nm was used for the measurement of tryptophyl fluorescence.

Fluorescence quantum yields, the efficiency of energy transfer and the temperature dependence of the tryptophyl fluorescence were determined at pH 7.0 in 0.05 M phosphate buffer containing 20 mM $CaCl_2$ as described in ref. 19.

Fluorescence lifetime measurements were carried out at 20 °C using a nanosecond single photon counting spectrofluorometer (System PRA 2000) and a nitrogen-filled flash lamp with a full width at half-maximum (FWHM) of about 2.5 ns. The data were analysed by convoluting the instrument response function $Y(t')$ with an assumed decay function $P(t)$

$$R_c(t) = \int_0^t Y(t')P(t-t')\,dt'$$

The $R_c(t)$ value obtained was compared with the experimental time dependence $R_m(t)$. The accuracy in the excited state lifetime (τ) determination was ± 0.2 ns. The decay curves contained 10^4 counts at the maxima. The time interval for these curves was 100 ps per channel.

3. Results and discussion

Table 1 summarizes the fluorescence parameters of the six subtilases. The main natural source of the emission in these proteins are indole and phenol groups. Tryptophans are both rare and, at the same time, conserved residues. Comparison of the amino acid sequences of the six proteinases from the microorganisms listed in Table 1 shows some conservation in the positions of these residues [10]. In the polypeptide chains of four proteinases (SBMEP, SBNOVO, SBCARL and SBDY) there is a tryptophan at position 113 [20–22]. In two cases (SBMEP and SBNOVO) positions 106 and 241 are also occupied by tryptophan residues [20, 21]. The tryptophans in SBTHER are located in different positions [23].

Usually, the emission from tryptophyl side-chains dominates the fluorescence of proteins containing both tyrosyl and tryptophyl chromophores. This spectroscopic rule is explained by a transfer of electronic excitation energy through the Forster

TABLE 1. Fluorescence parameters of subtilisins and subtilisin-like proteinases

Enzyme	Trp residues	Tyr residues	Emission λ_{max} (excitation at 300 nm) (nm)	Emission λ_{max} (excitation at 280 nm) (nm)	Trp emission quantum yield	Tyr-to-Trp energy transfer efficiency (%)	E_{act} (kJ)
SBMEP	3	12	342	342 (shoulder at 304 nm)	0.10	40	38
SBNOVO	3	10	342	342 (shoulder at 304 nm)	0.10	47	43
SBCARL	1	13	327	304	0.02	0	40
SBDY	1	14	329	304	0.03	0	43
SBTHER	6	15	332	332	0.10	70	67
SBPRK[a]	2	17	331	326	0.10	61	54

[a]Data from ref 19. Trp, tryptophan; Tyr, tyrosine.

mechanism from the phenol groups (donor) to the indole rings (acceptor). However, SBCARL and SBDY are exceptions to this rule. The fluorescence spectra of the two proteinases, obtained after excitation at 280 nm where both phenol and indole groups absorb, have a maximum at 304 ±1 nm (Fig. 1), in agreement with the values reported in refs. 16 and 24. This means that the two spectra are dominated by tyrosyl emission. SBCARL and SBDY contain a single tryptophyl residue (Trp 113) whose emission quantum yield is very low (0.02–0.03) (Table 1). Evidently the fluorescence of Trp 113 is quenched by the specific environment. Another reason for the absence of a tryptophyl component in the emission spectra of SBCARL and SBDY excited at 280 nm is the total lack of energy transfer from phenol groups to Trp 113

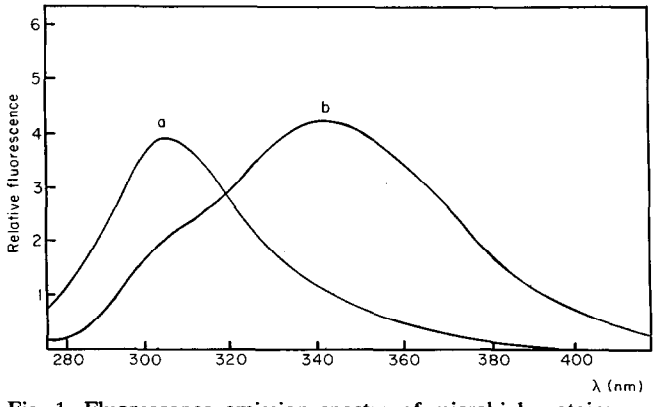

Fig. 1. Fluorescence emission spectra of microbial proteinases in 0.05 M phosphate buffer (pH 7.0) after excitation at 280 nm. Curve (a) represents the spectrum of PMS-SBDY or PMS-SBCARL; curve (b) is the spectrum of PMS-SBMEP or PMS-SBNOVO.

(Table 1), due to an unfavourable mutual orientation of the donor and acceptor groups,

Inspection of X-ray models of SBCARL, SBDY, SBNOVO and SBMEP in the region of Trp 113 shows an essentially identical microenvironment in the four proteins created by the side-chains of Ala 48, Ser 49, Gly 47, Asn 117, Val 93, Ala 114, Tyr 91 and Met 119 (Ala, alanine; Ser, serine; Gly, glycine; Asn, asparagine, Val, valine; Met, methionine). The last residue is substituted with leucine (Leu) 119 in SBDY and Val 93 is substituted by isoleucine (Ile) 93 in this enzyme, which does not change the character of the environment significantly. The indole ring of Trp 113 is located just below the surface in a "cavity", one side of which is formed by hydrophobic side-chains. This environment explains the observed λ_{max} at 327–329 nm in the fluorescence spectra of SBCARL and SBDY excited at 300 nm (Table 1). Such emission maximum positions are characteristic of tryptophyl residues in a hydrophobic environment. The side-chain of Tyr 91 lies close to the indole group, the distance between the CD2 atom of Trp 113 and the CH2 atom of Tyr 91 being 3.72 Å. A Tyr-to-Trp energy transfer can be expected after excitation with radiation absorbed by both phenol and indole groups. However, the crystal models show that, in the four subtilisins, the spatial orientation of the phenol ring and the indole group closely approaches that of perpendicularly oriented donor and acceptor dipoles.

The efficiency of electronic excitation energy transfer between a donor and an acceptor strongly depends on the mutual orientation of both dipoles. The probability of such an event between the

residues mentioned above is very low, confirmed by the observed lack of Tyr-to-Trp energy transfer in SBDY and SBCARL (Table 1). Thus the fluorescence of Trp 113 is significantly quenched in all four subtilisins and the main sources of emission in SBMEP and SBNOVO are Trp 241 and Trp 106. Willis and Szabo [25] have suggested that glutamic acid (Glu) 112 can account for the strong quenching of the Trp 113 fluorescence. However, the carboxyl group of this residue points in an opposite direction from the side-chain of Trp 113 and cannot be responsible for the quenching.

An emission maximum at 342 ± 1 nm and a shoulder at 304 nm were observed after excitation of neutral solutions of SBMEP and SBNOVO at 280 nm, where both phenol and indole groups absorb (Fig. 1). The value of λ_{max} (342 nm) for SBNOVO is in good agreement with the published data on the subtilisin BPN'/NOVO [24]. The position of the shoulder at neutral pH is typical of tyrosyl emission. Partial quenching of the phenol fluorophores can be attributed to Tyr-to-Trp resonance energy transfer which is highly efficient (Table 1).

A decrease in pH transforms this shoulder into a second peak in the emission spectrum of SBMEP (Fig. 2). In this respect the fluorescence spectrum of SBMEP is unique because a simultaneous emission from tyrosyl and tryptophyl residues is observed. Usually proteins which contain both indole and phenol fluorophores show only tryptophan emission as a result of Tyr-to-Trp transfer of electronic excitation energy. For SBMEP the efficiency of this process at a singlet level decreases at acidic pH as a result of conformational changes. No such effect of pH on the resolution of the tyrosyl and tryptophyl fluorescence of SBNOVO was observed.

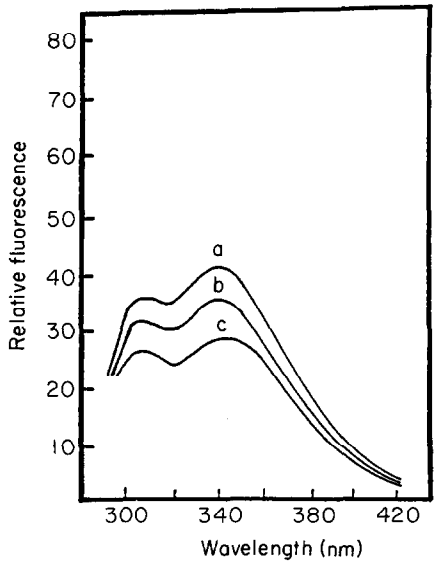

Fig. 2. Fluorescence emission spectra after excitation at 280 nm of SBMEP in 0.05 M citrate buffer at pH 4.8 (curve (a)), pH 4.6 (curve (b)) and ph 4.3 (curve (c)).

The emission spectra of the four subtilisins SBMEP, SBNOVO, SBDY and SBCARL, excited at 280 nm, offer a good opportunity to distinguish between subtilisins which belong to the subtilisin Novo group of alkaline proteinases and those which are similar to the Carlsberg enzyme (Fig. 1). The lack of Tyr-to-Trp 113 energy transfer in SBDY and SBCARL and the identical environment of this tryptophyl residue in the four subtilisins show that Trp 106 and Trp 241 in SBMEP and SBNOVO are the acceptors of a considerable part (40% and 47% respectively) of the incident light absorbed by the phenol groups. An exceptionally high degree of Tyr-to-Trp energy transfer was observed in SBTHER and SBPRK (70% and 61% respectively) (Table 1) due to the suitable mutual orientation of the donor and acceptor chromophores. The value of 47% calculated for the Tyr-to-Trp energy transfer in SBNOVO is in good agreement with that (52%) determined from the excitation spectrum of the diisopropylphosphoryl derivative of the BPN' enzyme [24].

Irradiation of SBMEP, SBNOVO, SBTHER and SBPRK with 300 nm radiation, where the tryptophyl side-chains are selectively excited, leads to fluorescence spectra with a maximum in the region 331–342 nm (Table 1). The emission maximum at 342 nm for SBMEP and SBNOVO is typical for "exposed" tryptophyl residues in a polar environment. Inspection of the respective X-ray models shows that Trp 106 and Trp 241 in both proteins are particularly "exposed" on the surface of the globule. Trp 106 is located in a "cavity"; it is surrounded by two segments of the polypeptide chain made up of residues 100–104 and 50–52. This indole group is near to the substrate-binding site, just behind Tyr 104. Trp 241 is located between the end of the central helix and the beginning of the N-terminal helix. In the model of SBMEP [10], the overall surface area of Trp 106, Trp 113 and Trp 241 accessible to solvent is 37, 48 and 49 $Å^2$ respectively. The degree of exposure of the three tryptophans in SBNOVO, which are located in identical positions in the polypeptide chain of this proteinase, is the same. These residues are accessible to the aqueous solvent, which explains the position of the emission maximum in the fluorescence spectra of the two proteinases. The main sources of emission are Trp 106 and Trp

241 because the fluorescence of Trp 113 is quenched by the specific environment.

It should be noted that the conclusions about the degree of exposure to the solvent of the tryptophyl residues in SBMEP, SBDY, SBCARL, SBNOVO, SBTHER and SBPRK are in agreement with previously performed quenching experiments [16, 19, 26, 27]. The emission maximum at 332 nm in the spectrum of SBTHER is typical of "buried" tryptophyl residues in a hydrophobic environment. In the X-ray model of this enzyme [15] the surface area accessible to solvent of tryptophans 24, 56, 112, 151, 199 and 266 is 4, 77, 58, 46, 49 and 0 $Å^2$ respectively. Trp 24 and Trp 266 are completely buried in the hydrophobic interior of the globule. Trp 56 is highly "exposed" on the surface and accessible to solvent. Trp 199 is partially "exposed". Trp 112 is in a position equivalent to that of Tyr 104 in the other subtilisins. The indole ring is located in a hydrophobic "cavity". Trp 151 lies near to the protein surface, in a small "cavity", one side of which is hydrophobic, and its side-chain points to the surface. It can be concluded from this description that the tryptophyl residues in SBTHER are in a predominantly hydrophobic local environment which can explain the emission maximum at 332 nm.

The 330 nm emission observed after excitation of SBPRK at 300 nm was attributed to exciplex formation between the excited indole ring and adjacent peptide bonds rather than to the hydrophobicity of the tryptophan environment, because the X-ray model showed a relatively high polarity of this environment [19].

Values of the activation energy E_{act} for the thermal deactivation of the excited indole groups in the six proteinases are given in the last column of Table 1. These values were obtained using PMS derivatives of the proteinases to avoid autolysis and in the presence of 20 mM $CaCl_2$ which stabilizes the subtilisin molecule. The value for SBMEP is in good agreement with published data [27, 28]. E_{act} is not very different for the four subtilisins.

The fluorescence decays of the six proteinases and Ac-Trp-NH_2 (N-acetyl-L-tryptophan amide) excited at 297 nm were well fitted by two exponentials. The theoretical fluorescence intensity at time t is given by the function $P(t) = A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2}$ where A_1 and A_2 are amplitudes. This function was used for the determination of the excited state lifetimes. Analysis of the data in terms of monoexponential models does not give a good fit between the experimental and theoretical curves. Figure 3(a) shows the decay of the fluorescence intensity of SBPRK. Similar curves were obtained for the other proteinases. The parameters which characterize the fluorescence decay functions of these proteins are presented in Table 2. The quality of the fit between the convoluted and experimental decay curves, and hence our confidence in the decay model and parameters found, was judged by inspection of weighted residual plots and by the determination of the parameter χ^2 [29]. In all cases the last parameter was less than or equal to 1.1. The distribution of the residuals between the theoretical and experimental decay curves and the shape of the autocorrelation function show a good fit, as is demonstrated for SBPRK in Figs. 3(b) and 3(c). Our instrumentation and techniques were tested by determination of the decay kinetics of the model compound Ac-Trp-NH_2. It is characterized by two lifetimes of 2.6 and 0.35 ns (Table 2). The first lifetime is in good agreement with the published value of 2.8 ns [30].

The fluorescence decays of the PMS derivatives of SBMEP and SBNOVO are described, to a good approximation, by two lifetimes. The lifetime of the long-lived component in the case of SBNOVO is 7.0 ns and that of the short-lived component is 1.1 ns, in excellent agreement with the data reported by Willis and Szabo [25]. These workers performed experiments with picosecond excitation and found that subtilisin BPN', identical with SBNOVO, shows a multiexponential decay. They determined two lifetimes in the nanosecond region of 7.9 ns (the major component) and 1.2 ns, as well as two lifetimes in the picosecond region of 253 and 55 ps.

SBMEP and SBNOVO are closely related proteinases with similar crystallographic structures [10]. Both proteins contain three tryptophyl residues located in identical positions in the respective polypeptide chains: 106, 113 and 241. Their steady state fluorescence characteristics are practically identical, the only difference being the presence of a second emission maximum at 304 nm in the emission spectrum of SBMEP at acidic pH (Fig. 2). The fluorescence decay properties clearly differentiate between the two proteins, which suggests that the respective tryptophans are affected differently by the local tertiary structure. The specific environment of the indole groups in SBMEP decreases the excited state lifetime by a factor of 2.4 in comparison with the value determined for SBNOVO. SBMEP has the shortest τ_1 value of 2.9 ns (Table 2). Comparison of the X-ray models of the two proteinases in the region of the tryptophyl residues shows differences in the environment of Trp 106 and Trp 241 in SBMEP in comparison with that in SBNOVO. Gly 97 in the

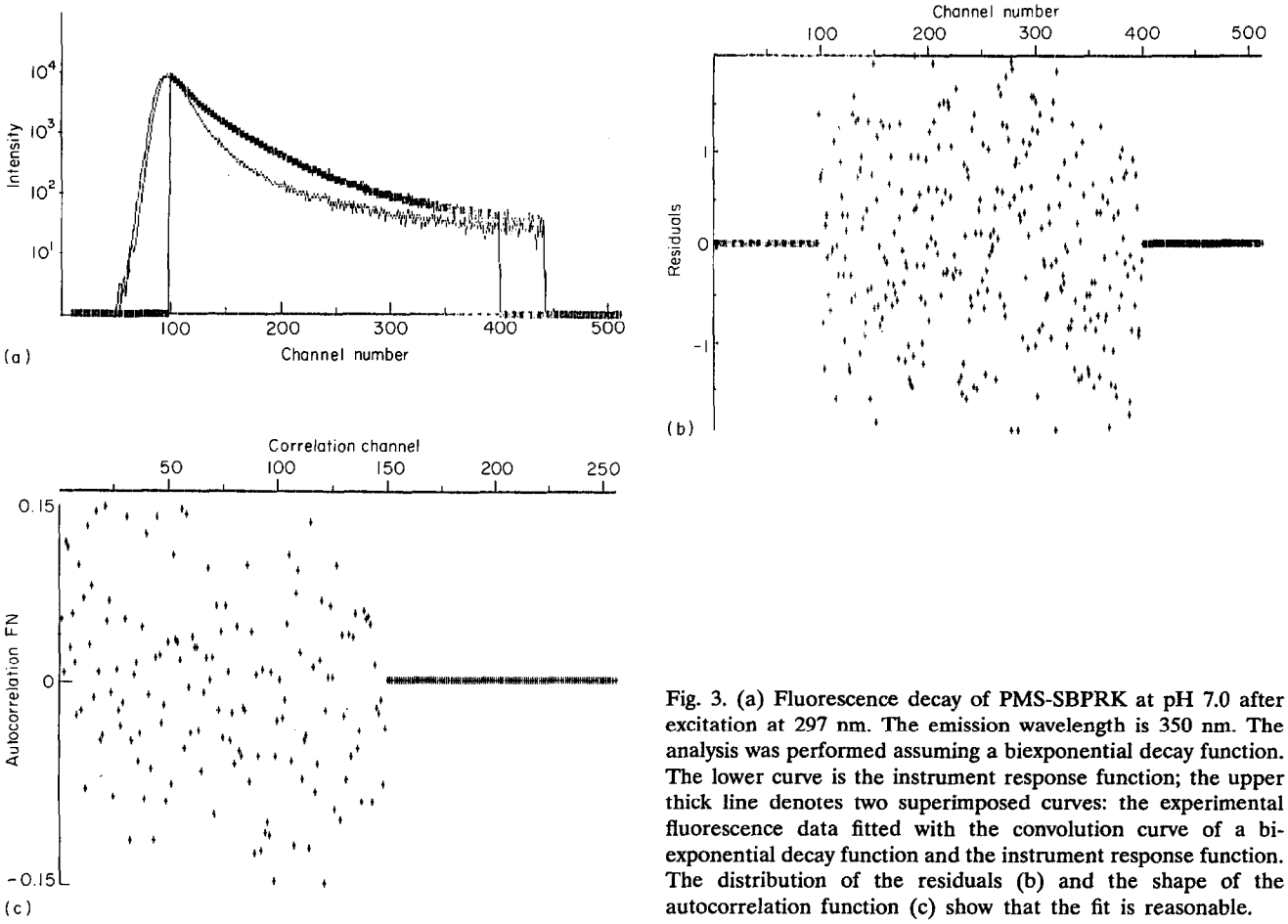

Fig. 3. (a) Fluorescence decay of PMS-SBPRK at pH 7.0 after excitation at 297 nm. The emission wavelength is 350 nm. The analysis was performed assuming a biexponential decay function. The lower curve is the instrument response function; the upper thick line denotes two superimposed curves: the experimental fluorescence data fitted with the convolution curve of a biexponential decay function and the instrument response function. The distribution of the residuals (b) and the shape of the autocorrelation function (c) show that the fit is reasonable.

immediate vicinity of Trp 106 in SBNOVO is substituted by aspartic acid (Asp) 97 in SBMEP. The distance between the CZ3 atom of Trp 106 and the OD2 atom of Asp 97 is 4.8 Å. The carboxyl group of the last residue participates in hydrogen bonds with threonine (Thr) 99 and the main chain nitrogen atom of the same residue. Protonated carboxyl groups can exert a quenching effect on the tryptophan emission. The immediate microenvironment of Trp 241 in SBNOVO is created by the side-chains of histidine (His) 238, the C-terminal carboxyl group of Glu 275, glutamine (Gln) 245, Thr 242, Ile 234 and Ser 249. All these residues, except the last, are present in the environment of the same tryptophan in SBMEP. Ser 249 in SBNOVO is substituted by arginine (Arg) 249 in SBMEP. The distance between the CZ3 atom of Trp 241 in SBMEP and the CG atom of Arg 249 is 3.2 Å. Trp 241 in both proteinases is surrounded by a system of hydrogen bonds. However, the hydrogen "network" in SBMEP is somewhat distorted in comparison with that in SBNOVO. All these changes in the environment of the two tryptophans can explain the decrease in the excited state lifetime of these residues in SBMEP.

TABLE 2. Fluorescence lifetimes (τ) of proteinases from microorganisms, relative amplitudes A and reduced χ^2

Enzyme	τ_1 (ns)	A_1 (%)	τ_2 (ns)	A_2 (%)	χ^2
SBMEP	2.9	19	0.55	81	1.05
SBNOVO	7.0	24	1.10	76	0.87
SBDY	3.5	28	0.50	72	0.91
SBCARL	3.7	30	0.90	70	0.90
SBPRK	3.5	60	0.60	40	1.10
SBTHER	5.3	41	1.28	59	1.05
Ac-Trp-NH_2	2.6	77	0.35	23	0.35

SBDY and SBCARL are closely related proteinases produced by different strains of *Bacillus subtilis* [16]. Both proteins contain a single tryptophan in position 113 of their polypeptide chains [22]. The long-lived lifetimes characterizing the decay kinetics of Trp 113 in the two enzymes are practically the same. The biexponential decay functions for the proteins containing a single tryptophyl residue can be explained by fluctuations in the

protein structure leading to a variability in the microenvironment of the indole ring [31] or by the existence of different rotamers of this residue. Different rotamers have different lifetimes because, for each rotamer, the distances of the quenching groups from the indole nucleus are different [32]. The emission of Trp 113 in the two subtilisins is affected in a similar manner by the specific environment.

However, there are some discrepancies from the published values for the fluorescence parameters of SBCARL and SBDY. Willis and Szabo [25] calculated three lifetimes for Trp 113 in SBCARL, the long-lived component being 3.3 ns. Our results ($\tau_1 = 3.7$ ns) are in agreement with this value. Bayley *et al.* [33] observed a complex fluorescence decay of the same residue in SBCARL with three to four exponential components and determined two lifetimes in the nanosecond region (5.2 and 1.7 ns) and two others in the picosecond region (266 and 70 ps). The first group [25] reported an emission maximum position at 322 nm as typical for SBCARL excited at 300 nm. The second group found a maximum at 331 nm under the same conditions [33]. Our experiments show an emission maximum at 327 nm after excitation at the same wavelength. Shopova *et al.* [34] found that the decay kinetics of Trp 113 in SBDY can be described by sums of three exponential terms, the longest lifetime being 5.3 ns. Inspection of the X-ray models of SBDY and SBCARL shows that the microenvironments of Trp 113 are practically identical. For this reason, the excited state lifetimes of this residue in the two proteinases should be similar if not identical. It is not mentioned in ref. 34 if special precautions were taken to avoid the effect of autolytic peptides which could explain the longer τ_1 observed by these workers. The discrepancies in the published data can also be explained by a confusion with regard to the authenticity of commercial preparations of subtilisins supplied for a period of about 10 years by Sigma and Serva [35]. Another reason may be differences in the instrumentation used. In the present study SBCARL and SBNOVO, obtained from the Carlsberg Laboratory, Copenhagen, were inhibited with PMSF, the autolytic products were discarded and the samples were checked for amino acid composition and homogeneity. The results reported here cannot be ascribed to the presence of autolytic products because we purified the PMS derivatives before the measurements. The abnormally low quantum yields of the samples of both SBCARL and SBDY (0.02 and 0.03 respectively) show that they are free of autolytic peptides. If the fluorescence observed originated mainly from tryptophyl residues included in such products, the emission quantum yield should be four to five times higher, comparable with that of Ac-Trp-NH_2.

The lifetimes calculated for SBPRK represent the fluorescence decay of Trp 8 and Trp 212. The individual contributions of the two indole rings to the total fluorescence were calculated to be 60% and 40% respectively [19]. The effect of the microenvironment on the excited state lifetime is similar to that observed in the cases of SBCARL and SBDY.

The lifetimes determined for SBTHER represent averages of the decay properties of six tryptophyl residues. The relatively high values of 5.3 and 1.28 ns show that the environment of these chromophores does not significantly quench their fluorescence emission.

The published data and the results presented here show that in some cases an accurate evaluation of the fluorescence parameters of subtilases is difficult and depends on the origin of the samples and the instrumentation used. In order to obtain comparable results, we have performed investigations on six proteinases using identical methods, instrumentation and experimental conditions for spectroscopic measurements. The observed fluorescence properties can be explained using the respective X-ray models.

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