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Resolution of Tyrosyl and Tryptophyl Fluorescence Emission from Subtilisins†

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ABSTRACT: Subtilisin Carlsberg is an exception to Teale's general rule [Teale, F. W. J. (1960) *Biochem. J. 76*, 381-388] that in proteins which contain both tyrosine and tryptophan residues the predominant contribution to the emission is from tryptophan [Longworth, J. W. (1971) in *Excited States of Proteins and Nucleic Acids* (Steiner, R. F., & Weinryb, I., Eds.) pp 319-484, Plenum Press, New York]. The tyrosyl and tryptophyl fluorescence contributions of underivatized subtilisin Carlsberg and the homologous enzyme subtilisin BPN′ were resolved in this study. Steady-state and picosecond time-resolved measurements over the whole emission spectrum were performed at different excitation wavelengths. Data were analyzed by using global techniques, and associated spectra of the exponential decay components were derived. Samples of subtilisin Carlsberg purified by a novel method and free of autolysis products were found to emit from *both* tyrosine and tryptophan at an excitation wavelength of 295 nm. There was evidence for a small tyrosine contribution in the emission from subtilisin BPN′ excited at 295 nm and in the emission from subtilisin Carlsberg excited at 300 nm. Careful purification of the enzymes is necessary in order to eliminate fluorescence from autolysis products.

Subtilisins are serine proteases produced by various species of *Bacillus*. Subtilisin Carlsberg (from *Bacillus licheniformis*) and subtilisin BPN′ (from *Bacillus amyloliquefaciens*) have been well studied (Markland & Smith, 1971), and their crystal structures are known at high resolution (Bode et al., 1987; Bott et al., 1988). The Carlsberg enzyme is used as a protein-digesting agent in detergents and is produced in greater quantities than any other industrial enzyme (Aunstrup et al., 1979). The BPN′ enzyme has been studied as a model system for protein engineering (Wells & Estell, 1988).

Subtilisin Carlsberg contains 274 amino acid residues differing from subtilisin BPN′ at 84 positions and by a deletion at Pro^{56} of BPN′. The former includes 1 tryptophan (Trp^{113}) and 13 tyrosine residues while the latter contains 3 tryptophan residues (Trp^{113}, Trp^{106}, and Trp^{241}) and 10 tyrosine residues (Markland & Smith, 1971). There is considerable sequence and structural homology between the two proteins which can assist in the interpretation of their fluorescence properties. Subtilisin Carlsberg displays unusual fluorescence characteristics. It is an exception to the general rule that in proteins which contain both tyrosine and tryptophan residues the overwhelming contribution to the emission is from tryptophan (Longworth, 1971). There has been some disagreement in the literature over the extent of tryptophan emission in subtilisin Carlsberg (Schlessinger et al., 1975; Brown et al., 1977). Since intrinsic fluorescence has been used to assess structural (Vaz & Schoellmann, 1976; Boteva et al., 1988) and dynamic (Bayley et al., 1987) properties of subtilisins, it is important that this is clarified. Fluorescence studies have been further complicated by the presence of autolysis products which may also contribute to the emission.

The purpose of this investigation was to resolve the tyrosyl and tryptophyl contributions to the emission of subtilisin Carlsberg and BPN′. This was achieved by combining the

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results of steady-state and time-resolved experiments to derive decay-associated spectra (DAS).[1] DAS represent the spectral distributions of each decay component and assist their assignment to a particular emitting species [Knutson et al., 1982; see Davenport et al. (1986) and references cited therein]. Lakowicz and Chenek (1981) used an analogous phase-sensitive detection technique to resolve the emission spectra of tyrosine or tryptophan in denatured human serum albumin. In this investigation, special consideration is given to the elimination of autolysis products from the samples.

Experimental Procedures

Proteins. Crystalline subtilisins Carlsberg (protease type VIII lots 87F-0490, 96F-0396, and 87F-0489) and BPN′ (protease "Nagarase" type XXVII lots 97F-0218 and 117F-0656) were purchased from Sigma. The PMS derivative of each enzyme was prepared by dissolving 17 mg of protein in a solution composed of 0.5 mL of 10 mM phosphate buffer, pH 7, and 0.1 mL of phenylmethanesulfonyl fluoride in dioxane (1.46 mg/mL). After 1 h at room temperature, the reaction mixture was dialyzed against 10 mM pH 6.2 phosphate.

Protein Purification. Protein samples were purified by an HPLC method based on procedures introduced by Polgar and Bender (1969). Typically, 17 mg of the crystalline protein was dissolved in 0.5 mL of 10 mM phosphate buffer, pH 6.2. The protein solution (or in the case of the PMS derivative 0.5 mL of the dialysate) was then immediately injected through a filter (Millipore 0.22 μm) onto the HPLC (Varian Vista 5500). A carboxymethyl ion-exchange column was used (TSK CM-3SW, 7.5 mm × 7.5 cm, 2700 theoretical plates) with the buffers A (10 mM phosphate, pH 6.2) and B (10 mM phosphate, pH 6.2, containing 0.08M NaCl. The sample was washed onto the column with buffer A (5 min) and then eluted by increasing the percentage of buffer B to 100% in a linear gradient over 35 min at a flow rate of 1 mL/min. The eluant was monitored at 280 nm. As the binding constants are high for calcium (Voordouw et al., 1976), the proteins are probably in their calcium-bound form. Enzyme purity and inactivation by PMSF were evaluated by titration with *N-trans*-cinnamoylimidazole according to the method of Bender (Bender et al., 1966). Protein concentrations were determined by using $\epsilon_{280} = 3.22 \times 10^4$ $M^{-1}\cdot cm^{-1}$ and $\epsilon_{280} = 2.34 \times 10^4$ $M^{-1}\cdot cm^{-1}$ for BPN′ and Carlsberg, respectively (Markland & Smith, 1971). All buffers were prepared from analytical-grade chemicals and double glass distilled, deionized water.

Spectroscopic Measurements. Time-resolved fluorescence measurements were performed by using the technique of time-correlated single photon counting with instrumentation described in detail elsewhere (Zuker et al., 1985). The excitation source was a cavity-dumped dye laser synchronously pumped by an actively mode-locked argon ion laser (Spectra Physics) operating at 825 kHz with a pulse width of 15 ps. Emission, following vertically polarized excitation at 295, 300, or 305 nm, was detected (right-angle geometry) after passing through a polarizer set at 55° to the vertical and a JY H10 monochromator, with a 4-nm band-pass, on a Hamamatsu 1564U-01 microchannel plate photomultiplier. The channel width was 21.6 or 10.8 ps/channel, and data were collected in 1024 channels. The instrument response function determined from a scattering solution of glycogen was typically four channels (full width at half-maximum, 21.6 ps/channel). Each decay curve typically contained 5×10^5 to 1.5×10^6 total counts and required 3–5 min of data collection. The ratio of laser pulses to single-photon events was 100:1 or greater. A "no protein" blank counts were measured for each sample for the same accumulation time, and the blank counts were subtracted from the sample decay curve.

The function describing the fluorescence intensity decay following δ-function excitation is assumed to be a sum of exponentials:

$$I(\lambda,t) = \sum_i \alpha_i(\lambda) \exp(-t/\tau_i) \quad (1)$$

where τ_i is the decay time of the ith component and $\alpha_i(\lambda)$ is its preexponential factor at emission wavelength λ. Since this model assumes τ_i is independent of λ, decay curves taken at different emission wavelengths can be analyzed simultaneously (Knutson et al., 1983). Data were analyzed by a global least-squares iterative convolution method based on the Marquardt (1963) algorithm. Adequacy of the exponential decay fitting was judged by inspection of the plots of weighted residuals and by the statistical parameters χ^2 [the reduced χ^2 (Bevington, 1969)] and SVR [the serial variance ratio (Durbin & Watson, 1971)]. Decay-associated emission spectra (DAS), the emission spectra associated with each individual decay component (Knutson et al., 1982), were calculated from

$$I_i(\lambda) = I_{ss}(\lambda)[\alpha_i(\lambda)\tau_i/\sum_i \alpha_i(\lambda)\tau_i] \quad (2)$$

where $I_i(\lambda)$ is the emission spectrum associated with the ith component, $I_{ss}(\lambda)$ is the total steady-state spectrum, and $\alpha_i(\lambda)\tau_i/\sum_i \alpha_i(\lambda)\tau_i$ is the fractional fluorescence of the ith component at wavelength λ.

Steady-state fluorescence measurements were made on an SLM 8000C spectrofluorometer in the ratio mode with polarizers oriented to eliminate anisotropic effects. Corrections were made for the signal from the appropriate blank and the wavelength dependence of the instrument response. For all fluorescence measurements, the absorbance of the sample at the excitation wavelength was less than 0.1, the sample temperature was 15 °C, and the band-pass was 4 nm. Absorption spectra were measured on a Varian DMS 200 spectrophotometer.

Results

HPLC. Typical results of ion-exchange HPLC of subtilisins Carlsberg and BPN′ are shown in Figure 1. The retention times for the corresponding PMS derivatives were identical since there was no significant increase in peak width when a mixture of equal concentrations of the native protein and PMS derivative was chromatographed. Rechromatography of the fraction corresponding to the main peak gave a single baseline-resolved symmetrical peak. Purified material that had stood at room temperature for 24 h gave a chromatograph similar to the commercial material, indicating that autolysis products are the major contaminants. Conventional carboxymethylcellulose ion-exchange chromatography (Polgar & Bender, 1969), while successful at removing material that eluted early on the HPLC system, was unable to resolve peaks that eluted close to the main peak. The HPLC method was also convenient and rapid; typically, 17 mg of commercial material gave, in 40 min, 2–3 mL of approximately 100 μM purified protein, which after suitable dilution could be used directly for fluorescence studies. Therefore, samples were prepared immediately before use, and an aliquot was rechromatographed at the end of the experiment to determine

[1] Abbreviations: PMS, phenylmethanesulfonyl; HPLC, high-performance liquid chromatography; SVR, serial variance ratio; DAS, decay-associated spectra.

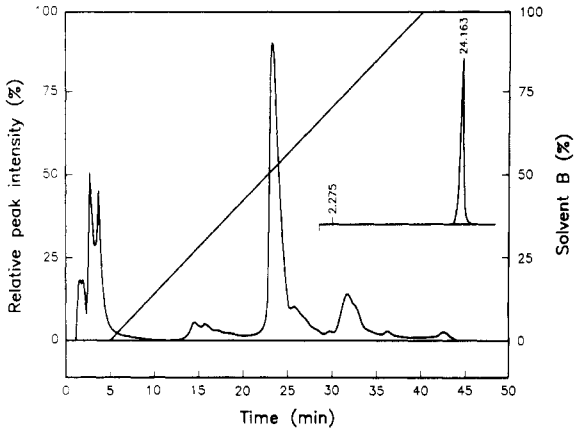

FIGURE 1: Ion-exchange HPLC of 17 mg of subtilisin BPN′ on a TSK CM-3SW column (7.5 mm × 7.5 cm). Solvent A was 10 mM phosphate, pH 6.2, containing 0.08 M NaCl. Chromatography was performed with a flow rate of 1 mL/min (6 atm pressure) at ambient temperature. Sample volume was 0.5 mL (in solvent A). The retention time of the protein was 23.6 min, and the protein peak comprised 41% of the total absorbance at 280 nm. Insert: Rechromatograph of 100 μL, 34 μM, of PMS subtilisin purified by HPLC. The peak with a retention time of 2.27 min occurred when buffer only was injected. Subtilisin Carlsberg gave a similar profile; the protein peak had a retention time of 32.9 min and comprised 62% of the total absorbance at 280 nm.

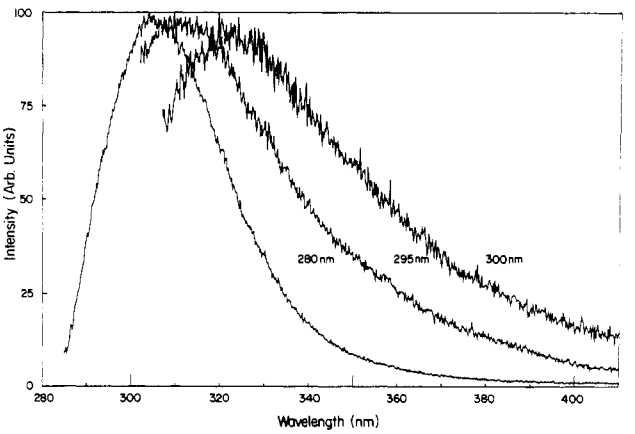

FIGURE 2: Dependence of the corrected emission spectra of HPLC-purified subtilisin Carlsberg on the excitation wavelength. The spectra were scaled to have equal maximum intensity by the factors 5.2 (λ_{ex} = 295 nm) and 10.6 (λ_{ex} = 300 nm). The buffer was 10 mM phosphate, pH 6.2, containing 63 mM NaCl, and samples had equal absorbance at the excitation wavelength.

Table I: Peak Fluorescence Emission Wavelengths (nm)[a]

sample	λ_{ex} = 280 nm	λ_{ex} = 295 nm	λ_{ex} = 300 nm
subtilisin Carlsberg[b]	305	315	340
purified subtilisin Carlsberg	304	311	322
subtilisin BPN′[c]	332	341	341
purified subtilisin BPN′	332	340	340

[a] Samples in 10 mM phosphate buffer, pH 6.2, containing 63 mM NaCl (Carlsberg) or 42 mM NaCl (BPN′). [b] Sigma lot 87F-0489. [c] Sigma lot 117F-0656.

the extent of autolysis. Under the conditions used for fluorescence measurements (15 °C, pH 6.2, and protein concentrations ≤50 μM), the underivatized proteins showed less than 1% autolysis over a 3-h period.

Titration of the purified native protein with *N-trans*-cinnamoylimidazole consistently gave percentage activities of between 95 and 96%.

Steady-State Fluorescence. The emission spectra of purified subtilisin Carlsberg showed a marked red-shift on increasing the excitation wavelength in the range 280–300 nm (Figure 2). There was no further red-shift in the emission maximum with excitation at 305 nm. Also noteworthy is the sensitivity of the peak emission wavelength (for excitation at 300 nm) on the purity of subtilisin Carlsberg, as presented in Table I. If a sample of purified subtilisin Carlsberg was allowed to autolyze, the emission spectrum (for excitation at 300 nm) underwent a red-shift, and the intensity of emission increased.

The steady-state emission properties of subtilisin BPN′ are essentially as reported by Schlessinger et al. (1975) and are relatively insensitive to purity (Table I). Within experimental error, the results for the PMS derivatives of subtilisins BPN′ and Carlsberg, which are inactive and unable to undergo autolysis, were identical with those observed for the native proteins.

Time-Resolved Fluorescence. The strong dependence of the emission spectra on excitation wavelength provided evidence for multiple emission sources. Time-resolved emission measurements were made to try to identify these sources and resolve their contribution to the total emission. The results of global analysis of decays from a series of emission wavelengths are given in Table II.

A three-exponential fit was required to describe the emission from subtilisin Carlsberg for excitation at 295 nm. Analysis of data taken at an excitation wavelength of 300 nm resulted in three similar lifetime components. This suggests that the same emission sources are being excited in each case, and reanalysis of the 300-nm data with the lifetimes "fixed" (i.e., varying only the preexponential factors) to the values from 295-nm excitation gave an acceptable fit. DAS resulting from 295-nm excitation are shown in Figure 3A. The long-lifetime component has a spectrum which peaks at about 305 nm characteristic of tyrosine emission (Longworth, 1971). The other two components peak in the region 320–330 nm. There is little change in the spectral shapes of the three components for excitation at 300 nm (Figure 3B), but the relative contribution of the long-lifetime component is considerably reduced. Time-resolved emission data at 325 nm for three excitation wavelengths (295, and 300, and 305 nm; Table II), when analyzed simultaneously, gave fractional fluorescences for the 3.3-ns component of 49 ± 0.02%, 25 ± 1%, and 23 ±

Table II: Results of Global Analysis to Time-Resolved Emission Spectral Data for HPLC-Purified Subtilisins[a]

sample	λ_{ex} (nm)	λ_{em} (nm)	no. of λ_{em}'s[b]	τ_1 (ns)[c]	τ_2 (ns)	τ_3 (ns)	τ_4 (ns)	χ^2	SVR
subtilisin Carlsberg	295	300–375	10	3.338 ± 0.003	0.197 ± 0.001	0.069 ± 0.001		1.08	1.76
	300	305–360	9	3.338	0.197	0.069		1.19	1.47
	295, 300, 305	325	1	3.299 ± 0.008	0.221 ± 0.003	0.086 ± 0.001		1.15	1.50
subtilisin BPN′	295	305–420	8	8.001 ± 0.01	2.448 ± 0.02	0.253 ± 0.003	0.055 ± 0.001	1.04	1.90
	300	310–430	10	7.897 ± 0.006	1.216 ± 0.03	0.130 ± 0.001		1.08	1.75
	300	310–430	10	8.001	2.448	0.253	0.055	1.03	1.93

[a] Samples in 10 mM phosphate buffer, pH 6.2, containing 63 mM NaCl (Carlsberg) or 42 mM NaCl (BPN′) at 15 °C. Preexponential factors are not given, but the relative contributions of each lifetime component can be assessed from the DAS. [b] Number of emission wavelengths used in the global analysis. [c] Lifetimes are given with their standard errors for recovery from a given global data set; where no standard error is given, analysis was performed with the lifetimes constrained to the stated values.

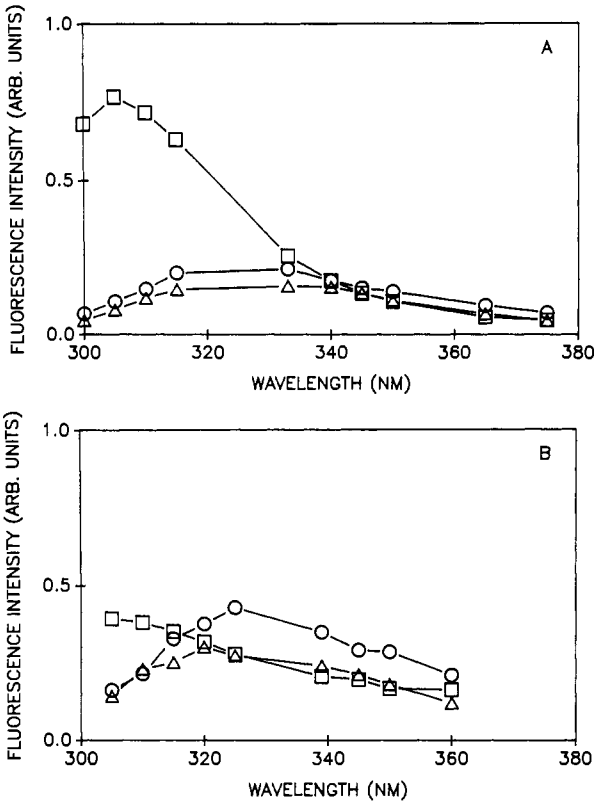

FIGURE 3: Decay-associated emission spectra for subtilisin Carlsberg. (A) Excitation at 295 nm. (B) Excitation at 300 nm. (□) 3.34-ns component; (○) 0.20-ns component; (△) 0.07-ns component. Spectra sum to the corresponding steady-state spectrum normalized to a value of 1 unit at the emission maximum. Errors are within the contours of the plotted symbols.

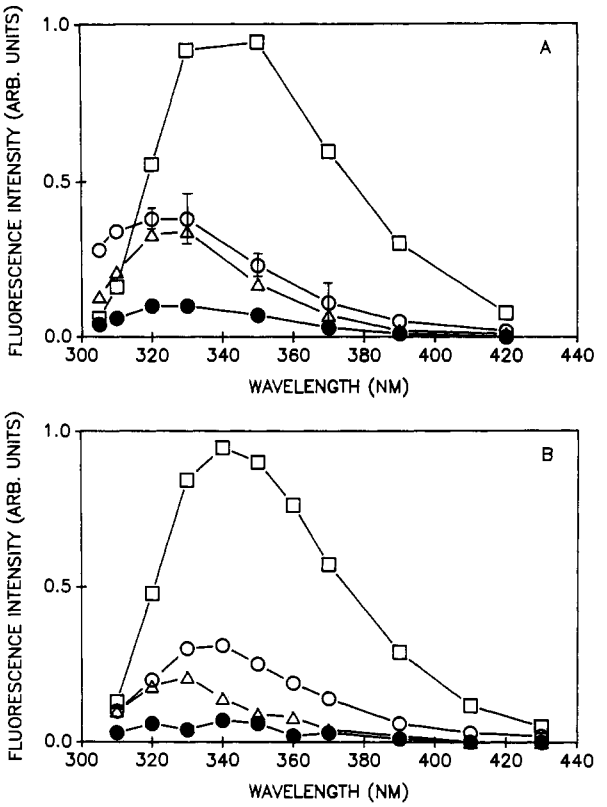

FIGURE 4: Decay-associated emission spectra for subtilisin BPN′. (A) Excitation at 295 nm. (B) Excitation at 300 nm. (□) 8.00-ns component; (○) 2.45-ns component × 10; (△) 0.25-ns component × 10; (●) 0.05-ns component × 10. Spectra sum to the corresponding steady-state spectrum normalized to a value of 1 unit at the emission maximum. Error bars are propagated errors computed from the standard errors of the fractional fluorescences. When no error bars are given, errors are within the contours of the plotted symbols.

2%, respectively. Therefore, even with 305-nm excitation, the 3.3-ns component remains a major fraction (23%) of the emission at 325 nm. The fractional fluorescences of the three lifetime components were similar for 300- and 305-nm excitation (at the emission wavelength of 325 nm) which is consistent with the steady-state result that the spectral shape of the total emission is unchanged.

An attempt to fit three decay terms to the data for subtilisin BPN′, excited at 295 nm, resulted in a poor fit (χ^2 = 1.26, SVR = 1.33). A four-component analysis, however, gave a satisfactory fit to the data (Table II). Three exponential terms were sufficient to fit the time-resolved emission data from 300-nm excitation of subtilisin BPN′, as judged by the statistical parameters alone. However, these data were also consistent with a four-component model in which the lifetimes were "fixed" to those determined from analysis of the 295-nm excitation data. Overfitting the 300-nm excitation data with an extra component allows direct comparison with the 295-nm excitation data. By analogy with the results for subtilisin Carlsberg, this may resolve a tyrosine contribution in the 295-nm excitation data. The DAS of subtilisin BPN′ emission (Figure 4) shows that the 8-ns lifetime component accounts for most of the emission (the three other components are shown scaled by a factor of 10 relative to the 8-ns component). The four-exponential analysis of the 300-nm excitation data was used to generate the DAS illustrated in Figure 4B. Similar spectra were obtained by using the three-component analysis results; effectively, the intensities of the third and fourth components were combined (not shown). Comparison of parts A and B of Figure 4 indicates that the spectrum of the 2.45-ns component is dependent on the excitation wavelength, the emission peak red-shifting from 320–330 nm for 295-nm excitation to 340 nm for 300-nm excitation. It is also apparent that the spectrum of the 2.45-ns component is less well-defined than that of the other components by the analysis of the 295-nm excitation data.

Discussion

Fluorescence spectroscopy has been used extensively to gain information on protein structure, function, and dynamics [see Taylor et al. (1986) and references cited therein]. Protein fluorescence is frequently complex, and meaningful interpretation often relies on the accurate resolution and assignment of individual emission components. This assignment may be compromised if the sample is impure. Therefore, we developed an HPLC technique to ensure a high degree of sample purity. However, chromatographic purity does not imply that the protein is all active enzyme. This was investigated by active-site titration with *N-trans*-cinnamoylimidazole. The titration gave a value of 0.95 active site per enzyme which probably indicates full activity within the limitations of the assay method.

We cannot exclude the presence of a small amount of oxidized subtilisin, since the oxidized enzyme is enzymatically active but with an efficiency less than that of the native enzyme (Stauffer & Eston, 1969). Recently, Bott et al. (1988) published the crystal structure of peroxide-oxidized subtilisin BPN′. This shows that in the crystal, the side chain of Trp^{113} is displaced as a result of oxidation of Met^{119}. However, we have found that the steady-state and time-resolved fluorescence properties of subtilisin Carlsberg and BPN′ were unaffected by oxidation with hydrogen peroxide prior to purification (unpublished results). Hence, if there is a small amount of

oxidized enzyme present, it does not affect the fluorescence results.

The subtilisins have a tendency to autolyze; consequently, spectroscopic studies are often performed on inactivated derivatives (Schlessinger et al., 1975; Brown et al., 1977). An advantage of the HPLC technique we have described is that samples can be prepared, and assayed for autolysis, rapidly. Under the conditions used in the fluorescence measurements, the proteins were autolytically stable for at least 3 h but were discarded after 1 h. HPLC assay confirmed negligible autolysis had occurred. The results of fluorescence measurements on the nonautolytic PMS derivatives of subtilisins Carlsberg and BPN′ were identical with those obtained from the native enzymes. This finding is in agreement with Genov et al. (1984).

An exception to Teale's (1960) general rule, that in proteins which contain both tyrosine and tryptophan and residues (class B proteins) the predominant contribution to the emission is from tryptophan, was noted by Longworth (1971). Longworth found that the main emitter in subtilisin Carlsberg was tyrosine. At an excitation wavelength of 295 nm, tryptophan emission from subtilisin Carlsberg could only be detected as a weak shoulder (350 nm) on a tyrosine-like spectrum. Schlessinger et al. (1975) reported that the emission maximum of PMS subtilisin Carlsberg was 350 nm for excitation at 300 nm, indicating that tryptophan emission predominates at this excitation wavelength. In contrast, Brown et al. (1977) found that subtilisin Carlsberg inactivated with diisopropyl fluorophosphate showed no evidence of tryptophan emission when excited at wavelengths from 280 to 302 nm. That denatured and autolyzed subtilisin Carlsberg displayed a small tyrptophan contribution for excitation wavelengths greater than 290 nm was also noted. Other workers have commented on the increase in tryptophan-like emission on autolysis of subtilisin Carlsberg (Boteva et al., 1981).

Our steady-state measurements confirm that the presence of autolysis products leads to an overestimation of the tryptophan contribution in subtilisin Carlsberg emission. This is presumably due to the release of tryptophan-containing peptides from a quenching environment in the native protein. Samples free of autolysis products have an emission maximum at 322 nm for excitation wavelengths of 300 and 305 nm (Table I). Since tyrosine absorption is negligible at these excitation wavelengths, the 322-nm emission is probably due to Trp^{113}. The shift in the emission peak to 311 nm on excitation at 295 nm indicates that tyrosine makes a significant contribution to the emission at this excitation wavelength. Excitation at 280 nm results in an emission spectrum which is dominated by tyrosine (Figure 2). In contrast, the steady-state emission properties of subtilisin BPN′ are relatively insensitive to both excitation wavelength and contamination with autolysis products. Our results for subtilisin BPN′ are in general agreement with those of other workers (Schlessinger et al., 1975; Brown et al., 1977).

Two groups[2] have reported results of time-resolved fluorescence studies on subtilisins. Grinwald and Steinberg (1976) found that the *p*-nitrobenzenesulfonyl derivatives of subtilisin Carlsberg had lifetimes of 4.9 and 1.6 ns for excitation at 296 nm and emission greater than 380 nm. These values must be viewed with caution as there have been considerable advances in instrumentation (O'Connor & Phillips, 1984) and data analysis (Knutson et al., 1983) since these measurements were made. Also, it has been reported that the *p*-nitrobenzenesulfonyl group results in quenching of subtilisin BPN′ fluorescence (Schlessinger et al., 1975). Bayley et al. (1987) measured fluorescence lifetimes and rotational correlation times for subtilisins Carlsberg and BPN′ at three emission wavelengths. However, commercial samples of the enzymes (Sigma) were used without purification, or consideration of the tendency of these enzymes to autolyze, and data were analyzed without deconvolution. For excitation at 295 nm, two lifetime components of 3.46 and 1.19 ns (average of three emission wavelengths) were reported for subtilisin Carlsberg. There is a much greater discrepancy between the results of this study (Table II) and those of Bayley et al. in the case of subtilisin BPN′. For example, subtilisin BPN′ (Sigma) used without purification was reported to have lifetimes of 4.54 and 1.29 ns (excitation 295 nm, emission 340 nm, sample in 0.1 M phosphate, pH 7.0 at 15 °C). When we repeated this measurement under the same conditions, also using unpurified material (Sigma lot 97F-0218), single-curve iterative convolution analysis gave an unacceptable fit to two components ($\chi^2 = 2$). A three-component fit was successful ($\chi^2 = 1.08$, SVR = 1.81), resulting in lifetimes of 7.16 ± 0.03, 1.65 ± 0.04, and 0.16 ± 0.005 ns with normalized preexponentials of 0.33, 0.22, and 0.45, respectively. These values are in reasonable agreement with our results for the purified material (Table II). To explain the discrepancy with the results of Bayley et al., we note that they report similar lifetimes for subtilisins Carlsberg and BPN′ (which is surprising in view of their differences in tryptophan content and steady-state spectra) and that until 1986 Sigma Chemical Co. was supplying subtilisin BPN′ that was in fact subtilisin Carlsberg (Russel & Fersht, 1986).

Steady-state and time-resolved data were combined to generate DAS, which represent the relative contributions of individual lifetime components, as a function of wavelength, to the total fluorescence. The information from DAS can be used to assign the lifetime components to individual (or groups of) emitting species. DAS for subtilisin Carlsberg excited at 295 nm (Figure 3A) show that the 3.3-ns component's spectrum has an emission maximum characteristic of tyrosine. The bandwidth, however, is greater than that normally observed for tyrosine. The lifetime probably represents an average value for the 13 tyrosine residues present in subtilisin Carlsberg and is close to the value of 3.76 ns reported for tyrosine at pH 6 (Laws et al., 1986). The molar extinction coefficients at 295 nm of the amino acids tyrosine and tryptophan in neutral aqueous solution are 30 and 2200 $M^{-1}\cdot cm^{-1}$, respectively, and the corresponding ratio of spectral quantum yields at 304 nm is 25:1 Tyr:Trp (Longworth, 1971). If a mixture of tyrosine and tryptophan in the ratio 13:1 (corresponding to the composition of subtilisin Carlsberg) was excited at 295 nm, 15% of the excitation would be absorbed by tyrosine, and at 304 nm, approximately 80% of the fluorescence intensity would be due to tyrosine emission. The free amino acids can be used as "first order" models of the extinction coefficients of tyrosine and tryptophan residues in proteins (Herskovits & Sorenson, 1968). Therefore, depending on the extent of quenching by the protein environment, tyrosine could make a significant contribution to the short-wavelength emission from subtilisin Carlsberg, excited at 295 nm. Figure 3A indicates that the tyrosine contribution is approximately 80% at 304 nm; this is largely fortuitous since the fractional contribution of tyrosine, to the total fluorescence, is also dependent on the quantum yield of the tryptophan emission. The significant tyrosine emission is consistent with low energy transfer to tryptophan,

[2] For completeness, we note that the results of lifetime measurements on subtilisin DY have recently been published (Shopova et al., 1987).

the high degree of solvent exposure (Brown et al., 1977), and the absence of disulfide bonds (Longworth, 1971). For excitation at 300 nm, the three-component fits are not as good as those to 295-nm excitation data ($\chi^2 = 1.19$ compared to 1.08, Table II). However, the fits are acceptable and give lifetime components consistent with the 295-nm excitation results. The 3.3-ns component makes a smaller contribution to the total fluorescence, and the bandwidth increases considerably (Figure 3B). With 305-nm excitation, where it can be reasonably assumed that the fractional absorbance of tyrosine is zero, the 3.3-ns component remains a significant fraction of the fluorescence. This suggests the long-lifetime component in Figure 3A,B results from contributions from tyrosine residues and an unresolved tryptophan component of closely similar lifetime. With 305-nm excitation, the 3.3-ns component is probably exclusively due to tryptophan emission. The 200- and 70-ps component spectra with maxima at approximately 325 nm are consistent with emission from a highly quenched tryptophan in a nonpolar environment. However, Trp^{113} of subtilisin Carlsberg is located in a relatively unrestricted environment near the surface of the protein and appears to be more "exposed" than Herskovits and Fuch's (1972) estimate of 30–40% exposure (for Trp^{113} of subtilisin BPN′) would suggest. We have examined the X-ray structure, and with the possible exceptions of Met^{119} and Glu^{112}, there appear to be no side chains in the region of Trp^{113} that could account for the strong quenching. Components with similar lifetimes and spectra have been reported for Trp^{172} and Trp^{215} in α-chymotrypsin (Desie et al., 1986). Most single-tryptophan proteins show multiexponential decay kinetics; the causes of the complex decays have been discussed by Beecham and Brand (1985) and may reflect conformational heterogeneity.

On the basis of quenching, X-ray, and solvent perturbation data, Shopova and Genov (1983) have suggested that in subtilisin BPN′, Trp^{113} is virtually nonfluorescent (like Trp^{113} in the Carlsberg enzyme), the "largely exposed" Trp^{241} contributes 20% of the fluorescence, and the "partially exposed" Trp^{106} accounts for the majority of the emission. A three-component fit could not satisfactorily describe the emission from subtilisin BPN′ excited at 295 nm. However, a four-component model with lifetimes of 8.00, 2.45, 0.25, and 0.05 ns resulted in excellent fits to both 295- and 300-nm excitation data. DAS of these four components (Figure 4) show that the emission is dominated by an 8-ns component with a spectrum characteristic of an exposed tryptophan. It is tempting to assign this to Trp^{106}; however, emission from this residue may be multiexponential; also, other fluorescent tryptophans could be contributing to this component. The X-ray structures of subtilisins Carlsberg and BPN′ are similar in the region of the Trp^{113}, and two "Carlsberg like" components are resolved in the BPN′ spectrum. The 2.45-ns lifetime in Figure 4B may result from Trp^{241}, but contributions from all three tryptophans cannot be excluded. Lakowicz (1983) has pointed out that dipolar relaxation of the protein matrix can also give rise to blue-shifted short-lifetime components in DAS. Comparing the DAS of the 2.45-ns component at the two excitation wavelengths (Figure 4), it is apparent that 295-nm excitation results in increased intensity in the region 300–330 nm. In addition, the 2.45-ns component is less well-defined when 295-nm excitation is used (Figure 4A). The results for subtilisin Carlsberg, discussed earlier, suggest that this may be due to the presence of a small tyrosine contribution. This is supported by the observations of Brown et al. (1977) that tyrosyl to tryptophyl energy transfer is only 50% efficient in subtilisin BPN′.

In conclusion, it is important to stress the complexity of the emission of these two homologous proteins. Subtilisin BPN′, for example, has 10 tyrosines and 3 tryptophans, each of which may (i) have distinct spectra, (ii) display multiexponential decay kinetics, and (iii) undergo excited-state reactions. We are at present realistically limited to a four-component model for these complex systems, and simplifying assumptions must be used. Many fluorescence studies of class B proteins have used 295 nm as an excitation wavelength in order to simplify the analysis by excluding the tyrosine contribution (Beechem & Brand, 1985). We have shown that the assumption that only tryptophan residues are excited at 295 nm is not always valid.

This work provides a basis for further fluorescence spectroscopic measurements on subtilisins. They are useful model proteins owing to their low tryptophan content, high degree of homology, and well-characterized structure and because engineered mutants are available (Wells & Estell, 1988). We were unable to fully account for the observed fluorescence properties on the basis of an examination of the high-resolution X-ray structures. The converse is also true which has implications for fluorescence studies of proteins of unknown structure. Extensive measurements on engineered mutant subtilisins could provide a better understanding of the relation between detailed protein structure/dynamics and fluorescence emission properties.

Added in Proof

Our suggestion that the "subtilisin BPN′" sample used in the Bayley et al. (1987) study was in fact impure subtilisin Carlsberg has been confirmed (P. M. Bayley, private communication).

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Reductive Dehalogenation of Chlorinated C_1-Hydrocarbons Mediated by Corrinoids†

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ABSTRACT: Corrinoids were found to catalyze the reductive dehalogenation of CCl_4 with either titanium(III) citrate or dithiothreitol as electron donors. $CHCl_3$, CH_2Cl_2, CH_3Cl, and CH_4 were detected as intermediates and/or products. In addition, several as yet unidentified nonvolatile compounds were formed. Ethane was a very minor product. The rate of dehalogenation decreased in the series CCl_4, $CHCl_3$, CH_2Cl_2, and CH_3Cl. Organocorrinoids were detected at completion of the reactions, suggesting that the dehalogenation involves the formation and reductive cleavage of alkylcorrinoids as intermediates. However, monoalkylcorrinoids cannot be the only intermediates in this process because the rate of methylcobalamin reduction to methane was much slower than the rate of methane formation from the chlorinated hydrocarbons catalyzed by either aquocobalamin or methylcobalamin. The present findings suggest that the reported slow reductive dehalogenation of CCl_4 and $CHCl_3$ by anaerobic bacteria may be catalyzed by corrinoids present in these microorganisms.

Biological dehalogenation of chlorinated hydrocarbons has been shown to occur via several mechanisms [for reviews, see Müller and Lingens (1986, 1987) and Cook et al. (1988)]. Chlorine can be removed as chloride anion by thiolysis or hydrolysis or by oxidative dehalogenation in the presence of oxygen. More recently, it has been demonstrated that in strictly anaerobic bacteria a reductive dehalogenation also occurs (Bouwer et al., 1981; Bouwer & McCarty, 1983; Vogel & McCarty, 1985; Belay & Daniels, 1987; Dolfing & Tiedje, 1987; Egli et al., 1987, 1989; Fathepure et al., 1987; Fathepure & Boyd, 1988; Bosma et al., 1988). Bouwer et al. (1981) reported that an undefined methanogenic mixed culture de-

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