Quenching of the Tyrosyl and Tryptophyl Fluorescence of Subtilisins Carlsberg and Novo by Iodide†

Michael F. Brown,‡ Siraj Omar, Rodger A. Raubach, and Thomas Schleich*

ABSTRACT: The tyrosyl and tryptophyl fluorescence of diisopropylphosphorylsubtilisins Carlsberg and Novo, respectively, is quenched efficiently by I^- but is not significantly affected by Cs^+. The I^- quenching data were analyzed using a modified Stern-Volmer treatment (Lehrer, S. S. (1971), *Biochemistry 10,* 3254), yielding values for the effective fraction of accessible protein fluorescence of 90–95 and 88–92% for the tyrosyl and tryptophyl emission of diisopropylphosphorylsubtilisins Carlsberg and Novo, respectively. Similar values were obtained at pH 5 and 7. The effective collisional quenching constant depends on pH in a manner suggesting the participation of protein surface charge in the quenching mechanism. Significant singlet energy transfer (efficiency = 0.52) from tyrosyl to tryptophyl residues was inferred from the excitation spectra of diisopropylphosphorylsubtilisin Novo. The very low efficiency of energy transfer to Trp-113 in diisopropylphosphorylsubtilisin Carlsberg suggests that Trp-105 and Trp-241 are the acceptors of tyrosyl emission in the homologous Novo enzyme. The unusually low quantum yield of Trp-113 in diisopropylphosphorylsubtilisin Carlsberg together with the tryptophyl fluorescence quenching behavior of the Novo enzyme suggests that this residue is "buried" and inaccessible to quenching in both enzymes. The tyrosyl quenching behavior of diisopropylphosphorylsubtilisin Carlsberg is consistent with the high degree of solvent exposure of aromatic residues evident in the x-ray model of subtilisin Novo.

Amino acid substitutions in homologous proteins can be used to advantage in spectroscopic studies which employ particular residues as intrinsic probes of structural detail. On the basis of circular dichroism (CD)[1] and secondary structure predictions, the details of polypeptide chain folding, internal side chain packing, and hydrogen bonding are thought to be conserved among the homologous subtilisins, with the exception of a sequence region containing the Pro-56 deletion in subtilisin Carlsberg (M. F. Brown and T. Schleich, manuscript in preparation). Although the crystal structure of subtilisin Carlsberg is not known, subtilisins Novo[2] and Carlsberg have been studied in solution using CD (Myers and Glazer, 1971; Brown and Schleich, 1975; M. F. Brown and T. Schleich, manuscript in preparation), infrared spectroscopy (Johansen and Ottesen, 1974), fluorescence spectroscopy (Longworth, 1971; Schlessinger et al., 1975), and solvent perturbation techniques (Myers and Glazer, 1971; Herskovits and Fuchs, 1972). These studies support extensive conformational homology among the subtilisins, although differences in the environments of the aromatic residues are evident.

In this paper we present a study of the I^- quenching of the tyrosyl and tryptophyl fluorescence of inactivated subtilisins Carlsberg and Novo, respectively. The use of polarizable salts such as I^- and Cs^+ to estimate the accessibility of protein fluorophores to solvent is well established (Lehrer, 1967; Burstein, 1968a,b; Lehrer, 1971). The presence of tryptophyl and tyrosyl substitutions among subtilisin enzymes makes them attractive for study by fluorescence techniques. In particular, only one of the three tryptophyl residues of subtilisin Novo (Trp-113) is retained in subtilisin Carlsberg (Trp-106 and Trp-241 are substituted by Gly and Leu, respectively), so that structural homology affecting the environment of Trp-113 can be studied from the relative fluorescence behavior of the two enzymes. The fluorescence results are compared with both the solvent perturbation experiments and the x-ray model of subtilisin Novo.

Materials and Methods

Subtilisins Novo and Carlsberg were inactivated with diisopropyl fluorophosphate (Dip-F) or phenylmethanesulfonyl fluoride ($PhCH_2SO_2F$) and purified as described previously (Brown and Schleich, 1975). *N*-Acetyl-L-tyrosineamide (*N*-Ac-L-Tyr-NH_2) was obtained from Sigma Chemical Co., St. Louis, Mo. Reagent grade salts were used. The KI solutions contained 10^{-4} M $Na_2S_2O_3$ to prevent oxidation and formation of I_3^- (Lehrer, 1971). Fluorescence spectra were obtained using a Hitachi Perkin-Elmer MFP-2A fluorimeter operated in the direct mode at an ambient temperature of 22–23 °C. The emission and excitation monochromators were calibrated using a solution of anthracene in benzene. Spectra were generally recorded from 250 nm to the secondary scattering peak or 500 nm. Relative quantum yields were determined by integration of the uncorrected fluorescence spectra with a planimeter.

Results

Fluorescence Quenching of Model Compounds. The contribution of collisional quenching to the deactivation rate of an excited state fluorophore is described by the Stern-Volmer (1919) law:

† From the Division of Natural Sciences, University of California, Santa Cruz, California 95064. *Received September 16, 1976.* This work was supported by Grant No. BMS 75-17114 from the National Science Foundation. This work constitutes part of a thesis submitted in partial fulfillment of the requirements for the Ph.D. degree of M.F.B. at the University of California, Santa Cruz.

‡ Present address: Department of Biophysical Chemistry, Biocenter of the University of Basel, CH-4056 Basel, Switzerland.

[1] Abbreviations used: CD, circular dichroism; Dip-F, diisopropyl fluorophosphate; Dip, diisopropylphosphoryl; Gdn·HCl, guanidine hydrochloride; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; $PhCH_2SO_2$, phenylmethanesulfonyl; *N*-Ac-L-Tyr-NH_2, *N*-acetyl-L-tyrosineamide; UV, ultraviolet.

[2] Subtilisins Novo and BPN′ are identical enzymes (Olaiten et al., 1968; Robertus et al., 1971; Drenth et al., 1972). We use the same subtilisin Novo when referring to this enzyme.

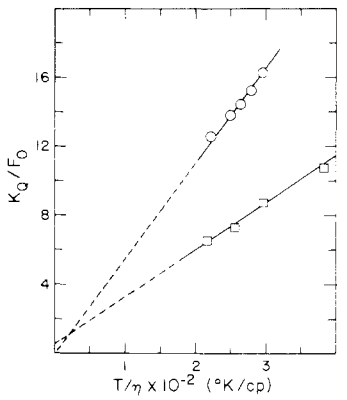

FIGURE 1: Dependence of the quenching constant, K_Q, on T/η for *N*-Ac-L-Tyr-NH_2 (0.02 mg/mL) in 0.5 M sodium acetate buffer, pH 7.0. Quencher (I^-) concentration varied from 0 to 0.25 M. K_Q was calculated using the simple Stern-Volmer equation (eq 1). Changes in viscosity were produced by the addition of sucrose (0–25%, w/v) (○) or by varying temperature (□).

$$F_0/F = 1 + K_Q[X] \tag{1}$$

where

$$K_Q = k_3\tau_0 \tag{2}$$

In the above expressions, F_0 ($= k_1/(k_1 + k_2) = k_1\tau_0$) is the quantum yield in the absence of quencher, where k_1 and k_2 are the rate constants for emission and radiationless deactivation, respectively, and τ_0 is the fluorescence lifetime; F is the quantum yield in the presence of quencher (X); and K_Q and k_3 are the quenching constant and bimolecular collisional rate constant, respectively. The quantity K_Q/F_0 is directly proportional to k_3 and yields a straight line when plotted vs. T/η, where η is the viscosity, for a collisional quenching mechanism (Vaughan and Weber, 1970; Lehrer, 1971).

The quenching of *N*-Ac-L-Tyr-NH_2 by I^- is shown in Figure 1. Within experimental error, a collisional quenching mechanism appears to be dominant. Similar conclusions have been drawn for tryptophyl model compounds (Burstein, 1968a; Lehrer, 1971).

Fluorescence Emission and Quenching of Dip-Subtilisins Novo and Carlsberg. The fluorescence emission of Dip-subtilisins Novo and Carlsberg at pH 5.0 in KCl and at a series of KI concentrations is shown in Figure 2. The fluorescence emission of Dip-subtilisin Novo at excitation wavelengths greater than 290 nm exhibits a single broad maximum at 342 ± 1 nm with a half bandwidth of 58–60 nm. These spectral parameters are characteristic of tryptophyl residue emission (Eisinger and Navon, 1969). The tryptophyl fluorescence of Dip-subtilisin Novo is efficiently quenched by I^-, whereas the cationic quencher Cs^+ was found to have negligible effect. No shift in the emission maximum was observed at I^- concentrations as high as 2.0 M. The fluorescence quenching behavior of Dip-subtilisin Novo is similar at pH values of 5 and 7, although quenching by I^- appears to be more effective at lower pH.

The fluorescence of Dip-subtilisin Carlsberg excited at wavelengths from 280 to 302 nm shows no evidence of tryptophyl emission and is attributed to the tyrosyl residues of the enzyme. The emission maximum occurs at 306 ± 1 nm and has a half bandwidth of 33 nm. The tyrosyl fluorescence of Dip-subtilisin Carlsberg is quenched efficiently by I^- (Figure 2) but is not quenched by Cs^+. No shift occurs in the emission maximum with increasing concentrations of KI. The fluorescence quenching behavior is similar at pH 5.0 and 7.0, with

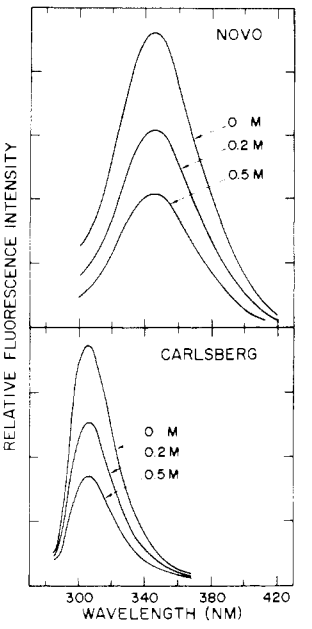

FIGURE 2: Fluorescence emission spectra of Dip-subtilisin Novo and Carlsberg in the presence of quencher (I^-). The solutions contained: 0.1 mg/mL protein, 0.25 M sodium acetate buffer, pH 5.0, KI (concentrations indicated in figure), and KCl (to maintain constant ionic strength, $I = 0.5$). λ_{ex} = 290 nm (Novo) and 280 nm (Carlsberg).

quenching more efficient at the lower pH. The tyrosyl fluorescence quenching behavior of this enzyme is in all respects similar to the tryptophyl fluorescence quenching of Dip-subtilisin Novo by I^-

The fluorescence quenching of proteins can be described by a modification of the Stern-Volmer equation (Lehrer, 1971), in which it is assumed that energy transfer between accessible and inaccessible fluorophores is negligible and that all fluorophores have identical absorptivities. The protein quantum yields in the presence and absence of quencher (F and F_0, respectively) are defined as:

$$F_0 = \frac{1}{n}\sum_{i=1} F_{0i} \tag{3}$$

$$F = \frac{1}{n}\sum_{i=1} F_i = \frac{1}{n}\sum_{i=1} \frac{F_{0i}}{1 + K_{Qi}[X]} \tag{4}$$

where n is the number of fluorophores of a given class, and F_{0i} and K_{Qi} are the quantum yield and quenching constant for the ith fluorophore. Assuming there are m accessible and $n - m$ inaccessible fluorophores ($K_{Qi} = 0$), then (Lehrer, 1971)

$$\frac{F_0}{F_0 - F} = \frac{F_0}{\Delta F} = \frac{1}{(f_a)_{eff}(K_Q)_{eff}[X]} + \frac{1}{(f_a)_{eff}} \tag{5}$$

For low quencher concentrations, a plot of $F_0/\Delta F$ vs. $[X]^{-1}$ is linear. The effective fractional maximum accessible fluorescence $(f_a)_{eff} = \Sigma K_{Qi}/\Sigma f_i K_{Qi}$, where $f_i = F_{0i}/\Sigma F_{0i}$, and $(K_Q)_{eff} = \Sigma K_{Qi}$ is the effective quenching constant.

Simple Stern-Volmer plots of F_0/F vs. $[I^-]$ (eq 1) for the I^- quenching of the fluorescence of Dip-subtilisins Novo and Carlsberg exhibit negative deviations from linearity at high concentrations of quencher, implying that the observed fluorescence is not completely quenched. Modified Stern-Volmer plots of $F_0/\Delta F$ vs. $[I^-]^{-1}$ (eq 5) are shown in Figure 3 for Dip- and $PhCH_2SO_2$-subtilisins Novo and Carlsberg at pH values of 5.0 and 7.0. The modified Stern-Volmer plots are linear over the range of I^- concentrations 0–0.5 M, suggesting that only a single class of tryptophyl or tyrosyl residues in Dip-subtilisins

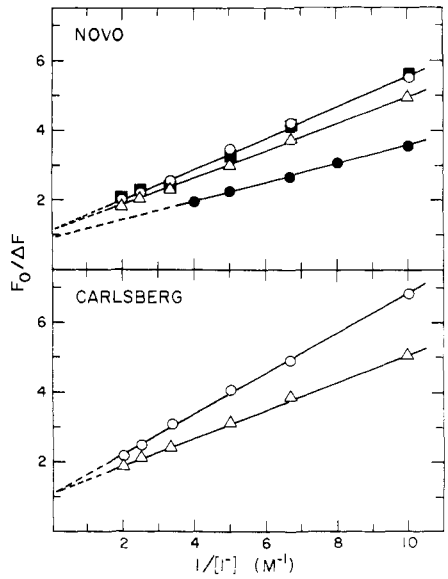

FIGURE 3: Modified Stern-Volmer plots of the fluorescence quenching of Dip- and $PhCH_2SO_2$-subtilisins Novo and Carlsberg. The solutions contained: 0.1 mg/mL protein, 0.25 M sodium acetate buffer, and KCl (to maintain constant ionic strength, I = 0.5); Dip-subtilisins Novo and Carlsberg at pH 7.0 (○); $PhCH_2SO_2$-subtilisin Novo at pH 7.0 (■); Dip-subtilisins Novo and Carlsberg at pH 5.0 (△); autolyzed subtilisin Novo at pH 7.0 (●).

TABLE I: Fluorescence Parameters for Subtilisins Derived from the Modified Stern-Volmer Equation (Equation 5).[a]

Protein Derivative	pH	$(K_Q)_{eff}$ (M^{-1})	$(f_a)_{eff}$ (%)
Dip-subtilisin Novo	5.0	2.92 ± 0.48	88 ± 5
Dip-subtilisin Novo	7.0	2.49 ± 0.17	89 ± 6
$PhCH_2SO_2$-subtilisin Novo	7.0	2.33 ± 0.23	92 ± 8
Dip-subtilisin Carlsberg	5.0	2.78 ± 0.12	90 ± 3
Dip-subtilisin Carlsberg	7.0	1.81 ± 0.14	95 ± 7

[a] Derived from an unweighted least-squares analysis.

Novo and Carlsberg, respectively, is selectively quenched. This is further supported by the lack of wavelength shift and the constant shape of quenched emission spectra at I^- concentrations as high as 2 M. Table I summarizes the values of $(f_a)_{eff}$ and $(K_Q)_{eff}$ derived from the slopes and intercepts of the modified Stern-Volmer plots (Figure 3); 88–92% of the tryptophyl fluorescence of Dip-subtilisin Novo is susceptible to I^- quenching (assuming eq 5). The effective collisional quenching constant is greater at pH 5.0 than at pH 7.0. Values of $(K_Q)_{eff}$ for Dip- and $PhCH_2SO_2$-subtilisin Novo at neutral pH are in good agreement. Approximately 90–95% of the Dip-subtilisin Carlsberg tyrosyl fluorescence is quenched by I^-, and this process is also more efficient at low pH. The I^- quenching of autolyzed subtilisin Novo in 6 M Gdn·HCl (Figure 3) reveals complete exposure of the tryptophyl fluorophores.

Tyrosyl–Tryptophyl Energy Transfer in Subtilisin Enzymes. Excitation spectra of $PhCH_2SO_2$-subtilisins Carlsberg and Novo at emission wavelengths of 306 and 400 nm are shown in Figure 4. Over this range of emission wavelengths, the uncorrected excitation wavelength maximum of $PhCH_2SO_2$-subtilisin Carlsberg shifts from 283–284 nm (λ_{em} = 306 nm) to 290 nm (λ_{em} = 400 nm), characteristic of tyrosyl and tryptophyl absorption, respectively (Horwitz et al., 1970). The excitation wavelength maxima of $PhCH_2SO_2$-subtilisin Novo are not as well separated as in subtilisin Carlsberg over the same range of emission wavelengths (λ_{ex} = 283–284 nm at λ_{em} = 306 nm and λ_{ex} = 287 nm at λ_{em} = 400 nm), suggesting the occurrence of appreciable energy transfer from tyrosyl to tryptophyl residues. Furthermore, the excitation maxima of autolyzed subtilisin Novo in 6 M Gdn·HCl (recorded at λ_{em} = 306 and 400 nm) are identical with those obtained for the native Carlsberg enzyme, indicating the presence of a tyrosyl excitation component in the tryptophyl emission of $PhCH_2SO_2$-subtilisin Novo.

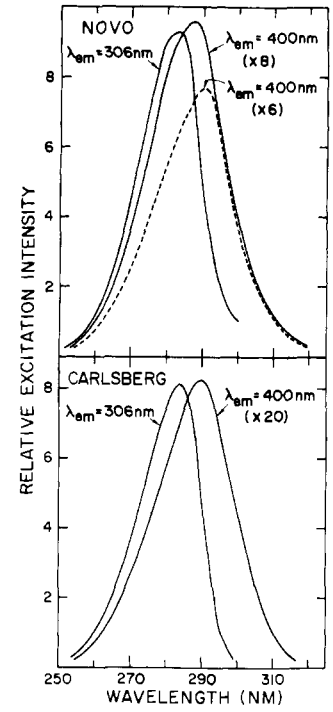

FIGURE 4: Fluorescence excitation spectra of $PhCH_2SO_2$-subtilisins Novo and Carlsberg (0.20 mg/mL) in 0.25 M sodium acetate buffer, pH 7.0, 0.5 M KCl (—), and autolyzed subtilisin Novo (0.20 mg/mL) in 6 M Gdn·HCl, 0.25 M sodium acetate buffer, pH 7.0, 0.5 M CKl (---). Emission wavelengths are indicated in the figure.

The emission spectral behavior of Dip-subtilisin Novo is also consistent with the occurrence of Förster energy transfer between the tyrosyl and tryptophyl residues of this enzyme. A series of emission spectra of Dip-subtilisin Novo at different excitation wavelengths is shown in Figure 5. At excitation wavelengths below 292 nm, a shoulder is present in the emission at 305 ± 1 nm indicative of tyrosyl residue emission (Teale, 1960, Konev, 1967; Eisinger, 1969). The overall efficiency of energy transfer (Förster, 1948, 1966) from tyrosyl to tryptophyl residues may be estimated from the following expression (Eisinger, 1969):

$$\phi_p(\lambda_{ex}) = \phi_{Trp}[f_{Trp}(\lambda_{ex}) + e_{Tyr}f_{Tyr}(\lambda_{ex})] \quad (6)$$

where

$$f_{Trp}(\lambda_{ex}) = \frac{n_{Trp}\epsilon_{Trp}(\lambda_{ex})}{n_{Trp}\epsilon_{Trp}(\lambda_{ex}) + n_{Tyr}\epsilon_{Tyr}(\lambda_{ex})} \quad (7)$$

and analogously for f_{Tyr}. The wavelength dependent quantum yield of protein fluorescence is $\phi_p(\lambda_{ex})$; ϕ_{Trp} is the average quantum yield of tryptophan in the protein and is wavelength independent; $f_{Trp}(\lambda_{ex})$ and $f_{Tyr}(\lambda_{ex})$ are the fraction of light

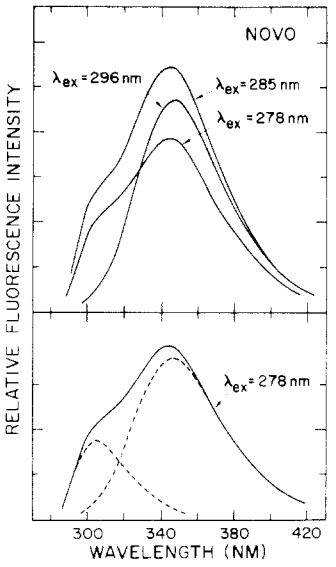

FIGURE 5: Fluorescence emission spectra of Dip-subtilisin Novo at different excitation wavelengths. The solutions contained: 0.1 mg/mL protein, 0.25 M sodium acetate buffer, pH 7.0, and 1.0 M KCl. Excitation wavelengths are indicated in the figure. The tryptophyl emission due to excitation at 296 nm was normalized to the fluorescence emission excited at 278 nm and subtracted to yield the resolved tyrosyl component (λ_{max} = 305 nm).

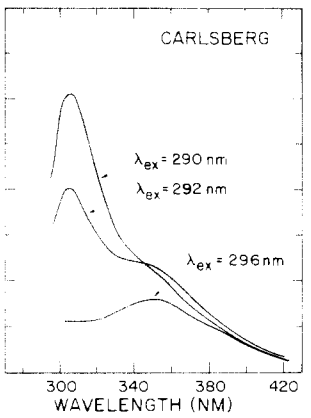

FIGURE 6: Fluorescence emission spectra of autolyzed subtilisin Carlsberg in 6.0 M Gdn·HCl. The solutions contained: 0.1 mg/mL protein, 0.25 M sodium acetate buffer, pH 7.0. Excitation wavelengths are indicated in the figure.

absorbed by tryptophyl and tyrosyl residues, respectively, calculated from eq 7; n_{Trp} and n_{Tyr} are the number of tryptophyl and tyrosyl residues, respectively; and $\epsilon_{Trp}(\lambda_{ex})$ and $\epsilon_{Tyr}(\lambda_{ex})$ are the wavelength-dependent molar extinction coefficients for tryptophyl and tyrosyl residues. The energy transfer coefficient is denoted by e_{Tyr} and represents the fraction of tyrosyl excitation energy transferred to tryptophyl residues. The fraction of light absorbed by phenylalanine is negligible above 275 nm and cysteine residues are not present. At excitation wavelengths above 292 nm, $f_{Tyr} \rightarrow 0$ and $f_{Trp} \rightarrow 1$. Therefore $\phi_p(\lambda_{ex}) \rightarrow \phi_{Trp}$ and we can use the protein quantum yield and number of tryptophyl residues to determine the average quantum yield of the tryptophyl residues in the protein. Using the 296 nm emission, we calculate a value of $\phi_{Trp} = 0.10 \pm 0.01$ for Dip-subtilisin Novo, which is slightly less than the fluorescence quantum yield for tryptophan model compounds in aqueous solution (0.13; Chen, 1967). The low value of ϕ_{Trp} is consistent with a weakly fluorescing component in the tryptophyl emission of Dip-subtilisin Novo. Using eq 6 and 7, we calculate a value of $e_{Tyr} = 0.52$ from the emission at either 278 or 285 nm.

Fluorescence Emission of Subtilisin Carlsberg in Gdn·HCl. In an attempt to observe the fluorescence of the single tryptophyl residue (Trp-113) of Dip-subtilisin Carlsberg quencher concentrations as high as 2.0 M were employed, and the sample was excited at the far-red edge of the tryptophan absorption envelope above 300 nm. Under such conditions, we found that the emission in the tryptophyl region is dominated by the Rayleigh and Raman scattering peaks, precluding unambiguous identification of any tryptophyl component. The fluorescence emission of autolyzed subtilisin Carlsberg in 6.0 M Gdn·HCl is shown in Figure 6 at different excitation wavelengths. The emission of Trp-113 is resolved as a shoulder at 350 nm when excited above 290 nm in Gdn·HCl.

Discussion

The fluorescence emission of Dip-subtilisin Novo excited at the red edge of the tryptophyl absorption envelope is characteristic of tryptophyl residues located in a polar protein environment (van Duuren, 1961; Walker et al., 1967; Eisinger and Navon, 1969). Energy transfer between some or all of the ten tyrosyl residues and one or more of the three tryptophyl residues is efficient (see below), with little tyrosyl emission observed at excitation wavelengths greater than 290 nm. The fluorescence emission of Dip-subtilisin Carlsberg, however, is due to tyrosyl residues at all pH values employed in this study, suggesting that the environment of the single tryptophan in Dip-subtilisin Carlsberg must be unfavorable for emission.

The relatively high quantum yield of tyrosyl fluorescence and the absence of significant tryptophyl emission in Dip-subtilisin Carlsberg suggests that Förster energy transfer from the tyrosyl residues to Trp-113 is absent or of very low efficiency. This is further supported by the observation of a pronounced separation between excitation spectra for light emitted at 306 and 400 nm (Schlessinger et al., 1975; this work). In this respect we note that Trp-113 is in close proximity to Tyr-91 in the x-ray model of Dip-subtilisin Novo, both of which are conserved in subtilisin Carlsberg. The phenol and indole rings of Tyr-91 and Trp-113 appear almost orthogonal in the x-ray model. A slight adjustment of the average crystal orientations of these residues in Dip-subtilisin Carlsberg would allow for an orthogonal orientation of the tyrosyl transition dipole moment with the plane of Trp-113, in which case resonance dipole coupling with the in-plane ^1L_a and ^1L_b electric transitions of Trp-113 could not occur. The other tyrosyl and tryptophyl residues are comparatively distant to Trp-113 in the x-ray model. The apparent lack of energy transfer between tyrosyl residues and Trp-113 in Dip-subtilisin Carlsberg suggests that Trp-106 and Trp-241 must be the exclusive acceptors of the tyrosyl excitation energy in Dip-subtilisin Novo.

Schlessinger et al. (1975) have observed a shoulder at ca. 350–360 nm in the fluorescence emission of $PhCH_2SO_2$-subtilisin Carlsberg which is attributed by them to the emission of Trp-113. We have repeated these experiments and have not observed any shoulders in our emission spectra for native Dip-subtilisin Carlsberg. However, a distinct shoulder at 350 nm is apparent in the emission of autolyzed subtilisin Carlsberg, suggesting that the discrepancy between our results and those of Schlessinger et al. (1975) may be due to the presence of autolysis products. Their conclusion of minimal energy transfer from tyrosyl residues to Trp-113 in $PhCH_2SO_2$-subtilisin Carlsberg is in agreement with our data.

Longworth (1972) has proposed that electronic energy transfer occurs in subtilisin Novo which is not completely efficient, in accord with our calculated efficiency of 0.52 for tyrosyl to tryptophyl energy transfer in Dip-subtilisin Novo.

The tryptophyl fluorescence of Dip-subtilisin Novo and tyrosyl fluorescence of Dip-subtilisin Carlsberg is efficiently quenched by I^- but not significantly affected by Cs^+. The magnitude of the effective quenching constants derived using a modified Stern–Volmer analysis of our data is in agreement with a collisional quenching model. Subtilisins Novo and Carlsberg are basic proteins (pI = 8.7 and 8.6, respectively) and are positively charged at the acidic and neutral pH values employed in this study. The pH dependence of $(K_Q)_{eff}$ in the range 5 to 7, and the lack of significant quenching by Cs^+ are consistent with the participation of protein surface charge in the collisional quenching mechanism. The selective quenching of the tyrosyl and tryptophyl fluorescence of Dip-subtilisins Carlsberg and Novo and the adherence to the modified Stern–Volmer law suggest that energy transfer from the accessible to inaccessible fluorophores does not occur.

Although the x-ray model of Dip-subtilisin Novo shows all of the tyrosyl and tryptophyl residues to be partially or completely exposed to solvent, a small but significant fraction of the tryptophyl fluorescence of Dip-subtilisin Novo and the tyrosyl fluorescence of Dip-subtilisin Carlsberg is not quenched by I^-. Since the near-UV CD of $PhCH_2SO_2$-subtilisin Novo is unchanged in 2 M KI, it is most unlikely that the high degree of fluorophore exposure arises from conformational alterations induced by I^-, a bipolymer destabilizing additive (von Hippel and Schleich, 1969). The I^- quenching experiments reveal that 88–92% of the tryptophyl fluorescence of Dip-subtilisin Novo and 90–95% of the tyrosyl fluorescence of Dip-subtilisin Carlsberg are subject to quenching. The fluorescence quenching of autolyzed subtilisin Novo in 6 M Gdn·HCl reveals complete tryptophyl exposure. By comparison, Lehrer (1971) has found that 38% of the native lysozyme fluorescence at pH 7.5 is amenable to quenching by I^-, which increases to 100% upon disruption of the protein structure by 6 M Gdn·HCl. The emission spectra of the tyrosyl and tryptophyl residues of Dip-subtilisins Carlsberg and Novo, respectively, suggest that the strongly fluorescing residues of these enzymes are highly exposed to the aqueous medium, and the lack of wavelength shifts in the emission spectra upon quenching by I^- concentrations as high as 2 M implies the absence of environmental heterogeneity. These observations are consistent with the high degree of solvent exposure for the aromatic residues in the x-ray structure of Dip-subtilisin Novo. Wavelength shifts and shoulders consistent with the presence of buried and inaccessible emission components have been observed for the I^- quenching of lysozyme (Lehrer, 1971) and the I^- and Cs^+ quenching of ribonuclease A (M. F. Brown, S. Omar, R. A. Raubach, and T. Schleich, unpublished observations).

The different mechanisms of fluorescence quenching and solvent perturbation together with the structural homology of subtilisins Novo and Carlsberg allow a more precise characterization of the accessibility of the tryptophyl residues of Dip-subtilisin Novo. Since the fluorescence quenching mechanism of these enzymes has been shown to be collisional, only a portion of the fluorophore need be exposed to solvent for quenching to occur. In light of the postulated structural homology of subtilisin enzymes (Wright et al., 1969; Kurihara et al., 1972; M. F. Brown and T. Schleich, manuscript in preparation), it is reasonable to expect a similar environment for Trp-113 in both Dip-subtilisins Novo and Carlsberg, although CD experiments suggest slight differences (M. F. Brown and T. Schleich, manuscript in preparation).[3] The high value of $(f_a)_{eff}$ (88–92%) obtained for Dip-subtilisin Novo is consistent with a low quantum yield and solvent inaccessibility of Trp-113 in this enzyme. The apparent inaccessibility of Trp-113 in Dip-subtilisin Novo cannot be due to clustering of acidic residues since none appear in this region of the protein. It is possible that solvent accessibility to Trp-113 of Dip-subtilisin Novo is blocked by an arrangement between the plane of this residue and Tyr-91 similar to that suggested for subtilisin Carlsberg. The side chain of Asn-117 and the polypeptide segment between Ala-45 and Ala-48 might also block solvent access to Trp-113. The I^- quenching experiments in conjunction with the solvent perturbation studies of Myers and Glazer (1971) and Herskovits and Fuchs (1972) suggest that tryptophyl residues 106 and 241 in subtilisin Novo have at least a part of their indole rings exposed to solvent.

The solvent perturbation data of Myers and Glazer (1971) suggest some exposure of Trp-113 and ca. 70% exposure of the tyrosyl residues of subtilisin Carlsberg to ethylene glycol, a value consistent with that obtained by Herskovits and Fuchs (1972) for active subtilisin Novo. The higher value of $(f_a)_{eff}$ (90–95%) obtained for Dip-subtilisin Carlsberg tyrosyl fluorescence is consistent with the different mechanisms of solvent perturbation and collisional quenching and suggests that one or two tyrosyl residues are buried. The unquenched tyrosyl fluorescence may arise from the abnormally ionizing tyrosyl residues of the enzyme (Markland, 1969; M. F. Brown and T. Schleich, manuscript in preparation).

Our conclusion of two partially exposed and one buried tryptophyl residue for Dip-subtilisin Novo is also supported by the analysis of Burstein et al. (1973), in which tryptophyl residues in proteins are grouped into classes depending on their fluorescence emission properties and half-bandwidths. Applying our data to their nomogram, we obtain a 0.6-mol fraction of the tryptophyl residues of Dip-subtilisin Novo in "class 1" or freely accessible to aqueous solvent and a 0.4-mol fraction located in a nonpolar environment. These results are in good agreement with our interpretation of the fluorescence quenching and solvent perturbation data.

Acknowledgment

We thank Randy Jolly for assistance in the construction of the subtilisin Novo model.

References

Brown, M. F., and Schleich, T. (1975), *Biochemistry 14,* 3069.

Burstein, E. A. (1968a), *Proc. Int. Conf. Lumin. 1966, 1,* 555.

Burstein, E. A. (1968b), *Biofizika 13,* 433.

Burstein, E. A., Vedenkina, N. S., and Ivkova, M. N. (1973), *Photochem. Photobiol. 18,* 263.

Chen, R. F. (1967), *Anal. Lett. 1,* 35.

Drenth, J., Hol, W. G. J., Jansonius, J. N., and Koekoek, R. (1972), *Cold Spring Harbor Symp. Quant. Biol. 36,* 107.

Eisinger, J. (1969), *Biochemistry 8,* 3902.

Eisinger, J., and Navon, G. (1969), *J. Chem. Phys. 50,* 2069.

Förster, Th. (1948), *Ann. Phys. (Leipzig) 2,* 55.

[3] Schlessinger et al. (1975) have arrived at similar conclusions from circular polarization of luminescence measurements.

Förster, Th. (1966), in Modern Quantum Chemistry, Sinanoglu, O., Ed., New York, N.Y., Academic Press, p 93.
Herskovits, T. T., and Fuchs, H. H. (1972), *Biochim. Biophys. Acta 263,* 468.
Horwitz, J., Strickland, E. H., and Billups, C. (1970), *J. Am. Chem. Soc. 92,* 2119.
Johansen, J. T., and Ottesen, M. (1974), *Isr. J. Chem. 12,* 339.
Konev, S. V. (1967), in Fluorescence and Phosphorescence of Proteins and Nucleic Acids, New York, N.Y., Plenum Press, p 69.
Kurihara, M., Markland, F. S., and Smith, E. L. (1972), *J. Biol. Chem. 247,* 5619.
Lehrer, S. S. (1967), *Biochem. Biophys. Res. Commun. 29,* 767.
Lehrer, S. S. (1971), *Biochemistry 10,* 3254.
Longworth, J. W. (1971), in Excited States of Proteins and Nucleic Acids, Steiner, R. F., and Weinryb, I., Ed., New York, N.Y., Plenum Press p 396.
Longworth, J. W. (1972), *Biophys. Soc. Abstr. 12,* 233a.
Markland, F. S. (1969), *J. Biol. Chem. 244,* 694.
Myers, B., II, and Glazer, A. N. (1971), *J. Biol. Chem. 246,* 412.
Olaiten, S. A., DeLange, R. J., and Smith, E. L. (1968), *J. Biol. Chem. 243,* 5296.
Robertus, J. D., Alden, R. A., and Kraut, J. (1971), *Biochem. Biophys. Res. Commun. 42,* 334.
Schlessinger, J., Roche, R. S., and Steinberg, I. Z. (1975), *Biochemistry 14,* 255.
Stern, O., and Volmer, M. (1919), *Phys. Z. 20,* 183.
Teale, F. W. J. (1960), *Biochem. J. 76,* 381.
van Duuren, B. L. (1961), *J. Org. Chem. 26,* 2954.
Vaughan, W. M., and Weber, G. (1970), *Biochemistry 9,* 464.
von Hippel, P. H., and Schleich, T. (1969), *Acc. Chem. Res. 2,* 257.
Walker, M. S., Bednar, T. W., and Lumry, R. (1967), *J. Chem. Phys. 47,* 1020.
Wright, C. S., Alden, R. A., and Kraut, J. (1969), *Nature (London) 221,* 235.

Effect of Oxidation of Methionine Residues in Chicken Ovoinhibitor on Its Inhibitory Activities against Trypsin, Chymotrypsin, and Elastase†

Yoram Shechter, Yigal Burstein, and Arieh Gertler*

ABSTRACT: Oxidation of methionine residues of chicken ovoinhibitor with *N*-chlorosuccinimide resulted in a selective loss of its inhibitory activities. While trypsin inhibiting activity was not affected at all, half of the chymotrypsin-inhibiting activity and all of the elastase inhibiting activity were lost. Electrophoretic and affinity chromatography studies indicated that the 50% loss of the chymotrypsin-inhibiting activity resulted from the inactivation of one of its two chymotrypsin-inhibiting sites rather than from a decrease in the binding constants of both sites. Oxidation of ovoinhibitor–chymotrypsin and ovoinhibitor–elastase complexes with excess of *N*-chlorosuccinimide indicated that the complex formation in each case protected the site that binds the enzyme which participated in the complex, but did not protect the site that binds the other enzyme. Quantitative estimation of the number of oxidized methionine residues in the ovoinhibitor isolated from the complexes has shown that in each complex about one methionine residue was protected from oxidation. Nitrophenylsulfenylation of the single tryptophan residue of ovoinhibitor did not affect its inhibitory activities at all.

In the last decade a considerable progress toward understanding the nature of interaction between proteolytic enzymes and their naturally occurring high molecular weight inhibitors has been made. Two main research approaches have been used: one consisted of limited proteolysis of trypsin and chymotrypsin inhibitors by the respective enzymes at acidic pH, which lead to a specific split of a single peptide bond related to the specific binding site (for review, see Laskowski and Sealock, 1971); a second more recent one was based on direct crystallographic analysis of the complex and its components (Ruhlmann et al., 1973; Janin et al., 1974).

Chicken ovoinhibitor, an egg-white glycoprotein, is one of the most complex naturally occurring polyfunctional inhibitors of proteinases. It is much larger in size than most other known trypsin inhibitors from plants and animals, and it is capable of inhibiting simultaneously 2 mol of trypsin and 2 mol of chymotrypsin (Tomimatsu et al., 1966) per mol of inhibitor. It also inhibits other proteolytic enzymes such as subtilisin, alkaline proteinases from *Aspergillus oryzae, Streptomyces griseus* (Davis et al., 1969; Liu et al., 1971), *Aspergillus soyae,* and porcine elastase (Gertler and Feinstein, 1971), although it is not entirely clear whether the binding sites for all these enzymes are distinct from the binding sites of chymotrypsin.

Ovoinhibitor appears therefore to be one of the most versatile proteinase inhibitors and presents an excellent case for

† From the Department of Organic Chemistry, The Weizmann Institute of Science, Rehovot, Israel (Y.S. and Y.B.), and the Department of Agricultural Biochemistry, Faculty of Agriculture, The Hebrew University Rehovot, Israel (A.G.). *Received July 8, 1976.* A preliminary report was presented at the annual meeting of the Israel Biochemical Society, 1976. This research was supported in part by Grant No. AMO5098 from the National Institutes of Health, United States Public Health Service.