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A Study of Subtilisin Types Novo and Carlsberg by Circular Polarization of Fluorescence†

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ABSTRACT: The circular polarization of the luminescence of a chromophore, in addition to its circular dichroism and optical rotatory dispersion, is a manifestation of its asymmetry. In the study of proteins, the circular polarization of luminescence yields more specific information than circular dichroism or optical rotatory dispersion since nonfluorescent chromophores do not contribute, and the spectra of the tyrosine and the tryptophan residues are much better resolved in emission than in absorption. The circular polarization of the fluorescence of the tyrosine and tryptophan residues in derivatives of subtilisin Carlsberg and subtilisin Novo were indeed resolved in this study. The tyrosine residues in the Carlsberg protein, and both tyrosine and tryptophan residues in the Novo protein, were found to be heterogeneous with respect to their optical activity and emission spectra. Changes in the environment of the emitting tyrosine residues in both proteins and in the tryptophan residues in the Novo protein were found on changing the pH from 5.0 to 8.3. The pH dependence of the enzymatic activity of these proteins may thus be due, at least in part, to conformational changes in the molecules. Fluorescence circular polarization also revealed that covalently bound inhibitors at the active site of subtilisin Novo affect the environment of the emitting aromatic side chains, presumably *via* changes in conformation.

A wide variety of spectroscopic techniques have contributed to our growing understanding of the role played by macromolecular conformation in the molecular biology of proteins. Among these techniques, circular dichroism, CD, has helped to elucidate some of the details of the secondary and tertiary structure of proteins. CD is an absorption phenomenon arising from the preferential absorption of right- or left-handed circularly polarized light by an optically active chromophore which may be either intrinsically asymmetric or perturbed by an asymmetric environment. In the case of proteins several chromophores, each involving one or more electronic transitions in the experimentally accessible spectral range, can contribute to the observed CD spectrum.

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These include the peptide bonds, S–S cross-links, and aromatic side chains. All of these chromophores derive their CD properties from the asymmetric perturbations arising from the L configution of the α-carbon atom of the primary covalent chain structure, the α helical and other secondary structures, and the folding of the polypeptide chain into a variety of asymmetric tertiary structures. The spectral overlaps among the absorption bands of the various chromophores in proteins and the complex contributions to the observed CD arising from the asymmetric perturbations of the chromophores by the primary, secondary, and tertiary structures, have made it very difficult to recover all the structural information contained in the CD spectra of proteins.

Recently, it has been shown that the optical activity of an asymmetric molecule is manifested in an additional spectroscopic property if the molecule is luminescent. The light emitted by asymmetric luminescent molecules may be partially circularly polarized (Emeis and Oosterhoff, 1971; Gafni and Steinberg, 1972; Schlessinger and Steinberg, 1972; Steinberg, 1974; Steinberg *et al.*, 1974). Circular polarization of luminescence, CPL,[1] is the emission analog of CD and is related to the molecular conformation in the electronically excited state in the same way that CD is related to the molecular conformation in the ground state (Steinberg, 1974; Steinberg *et al.*, 1974). The spectral characteristics of CPL make this spectroscopic method a very useful tool for the exploration, in more subtle detail, of the conformational behavior of peptides, polypeptides, proteins, and protein–ligand complexes (Schlessinger and Steinberg, 1972; Gafni *et al.*, 1973; Schlessinger *et al.*, 1974; Steinberg *et al.*, 1974; Veinberg *et al.*, 1974; Schlessinger and Levitzki, 1974; Gafni and Steinberg, 1974). While there is an analogy between CD and CPL, it should be pointed out that the additional information obtained from the measurement of CPL is not redundant and in some cases may indeed be unique. This point is illustrated for the case of proteins in the following.

Since only luminescent molecules contribute to the CPL spectrum, the information obtained has more specificity than that yielded by CD. For proteins this means that the conformation of the molecule is probed by CPL only in the region of those Trp and Tyr residues which are both fluorescent and are situated in an asymmetric environment when they are in their electronically excited states (Steinberg *et al.*, 1974). The spectral resolution between tyrosine and tryptophan, and among different residues of each type, is significantly greater in emission than in absorption. In condensed media, and hence also in proteins, CPL is usually associated with a single electronic transition ($S_1 \rightarrow S_0$) whereas, for example, the tryptophan chromophore is associated with two transitions (from the ground state to the excited states ^1L_a and ^1L_b) in its last absorption band (Weber, 1960; Yeargers, 1968; Strickland and Billups, 1973). It is thus clear that the CPL of proteins may provide specific information which may be difficult to deduce from their CD spectra.

The analysis of CPL spectra is greatly facilitated by application of a theorem formulated originally by Moscowitz for CD (Moscowitz, 1965) and which is equally applicable to CPL (Steinberg, 1974; Steinberg *et al.*, 1974). For a CD band due to a single electronic transition the magnitude of $\Delta\epsilon/\epsilon$ is expected to be approximately constant across the band, provided the transition is an allowed one ($\Delta\epsilon$ being the difference between the absorption coefficients for circularly polarized light of opposite sense and ϵ being the average absorption coefficient). Analogously, the extent of circular polarization of the luminescence, $\Delta f/f$, for a single allowed transition is expected to be approximately constant across the corresponding band. As mentioned above, the fluorescence emitted from a pure compound in condensed phases involves as a rule a single electronic transition, and should thus exhibit an approximately constant value for $\Delta f/f$ across the whole of its emission band. Marked deviation from such behavior may therefore serve as a strong indication of heterogeneity of emitting chromophores in the system studied; the different chromophores having different emission spectra and different optical activity, thus causing $\Delta f/f$ to assume different values across the observed composite emission spectrum. The heterogeneity in the emitting chromophores may stem from the presence of different kinds of emitting species, such as tyrosine and tryptophan residues in a protein, or differences in the environment of chromophores of the same kind, such as the various tryptophan residues in a protein molecule. As will be seen below, this aspect of CPL renders this technique a rather powerful tool for the discrimination among various fluorescing groups in protein molecules.

Most proteins which contain both Tyr and Trp residues show predominantly tryptophan fluorescence as a result of the relatively low quantum yield of Tyr in the macromolecule and efficient long range transfer of electronic excitation energy from electronically excited tyrosine to tryptophan. The case of subtilisin types Novo and Carlsberg is an exception to this rule (Longworth, 1971, 1972) and the fluorescence of both tyrosine and tryptophan residues is observable when the molecule is excited in the tyrosine absorption band, presumably because the usual mechanisms leading to the quenching of tyrosine fluorescence are less efficient than usual (R. S. Roche and M. Shinitzky, in preparation). These proteins therefore provide a unique opportunity for the study of tyrosine and tryptophan fluorescence, and particularly their CPL, in the same molecule.

The subtilisins are sequence-homologous serine proteases of bacterial origin (Ottesen and Svendsen, 1970). They are single polypeptide chains without disulfide bonds. The three-dimensional structure of subtilisin Novo, which has 275 amino acid residues in the molecule, is known at a resolution of 2.5 Å (Wright *et al.*, 1969). Subtilisin Carlsberg differs from subtilisin Novo at 85 positions, including one deletion. The sequence homology suggests that there may be extensive conformational homology although there is some evidence to suggest that there are differences in conformation in the region of Trp-113 (R. S. Roche and M. Shinitzky, in preparation). The subtilisins are autolytic. Although autolysis is not rapid, we have worked exclusively with inactive derivatives of these enzymes. In order to explore the question of whether the formation of active site derivatives significantly perturbs those parts of the molecule probed by the CPL technique, namely the regions where Tyr and Trp residues are located, we prepared thiol-subtilisin Novo which is not autolytic and which has a tertiary structure identical with the naturally occurring enzyme (Kraut *et al.*, 1969). The fluorescence properties of thiol-subtilisin are similar to those of subtilisin Novo (Neet *et al.*, 1968).

The results of this study show that in the case of the sub-

[1] Abbreviations used are: $PhCH_2SO_2$, phenylmethanesulfonyl; nisyl, *p*-nitrobenzenesulfonyl; CPL, circular polarization of luminescence.

tilisins the separation of tyrosine and tryptophan emission observed in their fluorescence can be further resolved by the CPL technique, and that useful insights can be gained about the effect of pH and protein denaturants on the local and gross conformational structure of these molecules.

Materials and Methods

Materials. Subtilisin Novo and subtilisin Carlsberg (Novo Industries, Copenhagen, Batch No. 120-2 and 70-3, respectively) were purified according to Ottesen and Svendsen (1970). Sephadex G-25 chromatography, 0.01 M phosphate buffer at pH 7.0 at 5°, was followed by separation from the esterase impurity on a carboxymethyl-cellulose column (CMC-23) applying a linear salt concentration gradient, starting with 0.01 M phosphate and ending with 0.1 M phosphate, both at pH 7.0. The second and larger of the two peaks, monitored at 280 nm, emerging from the latter column was isolated, dialyzed exhaustively against distilled water at 5°, and lyophilized. The $PhCH_2SO_2$ derivative of each enzyme was prepared by reaction with phenylmethanesulfonyl fluoride using procedures previously described (Polgar and Bender, 1966). This procedure was also followed for the synthesis of the thiol derivative of subtilisin Novo (thiolsubtilisin-Novo).

The nisyl derivative of both enzymes was prepared by procedures previously described (Kallos and Avatis, 1966). The purity of each enzyme preparation and the completion of the derivatization reactions were evaluated in each case by titration with *N-trans*-cinnamoylimidazole according to the method of Bender (Bender *et al.*, 1966). Protein concentrations were determined using $E_{280\,nm}$(1 mg/ml) 0.96 and 1.17 for subtilisin Carlsberg-$PhCH_2SO_2$ and subtilisin Novo-$PhCH_2SO_2$, respectively (Ottesen and Svendsen, 1970).

Fluorescence and CPL Measurements. The instrument for the CPL measurements was built in our laboratory and has been described elsewhere (Steinberg and Gafni, 1972; Gafni *et al.*, 1973; Steinberg *et al.*, 1974). The light source is a 100-W high-pressure mercury lamp (Osram HBO 100W/2). The wavelength of the excitation light was selected by a Bausch and Lomb high intensity monochromator which was set at 254 or 275 nm with a band pass of 30 nm. Stray light was removed by a chemical filter composed of two solutions: (1) 60 g of $NiSO_4$ in 100 ml of water; 2-cm light path, and (2) 5 mg of cyanine (2,7-dimethyl-3,6-diazacyclohepta-1,6-diene perchlorate, K and K Laboratories, Inc. production) dissolved in 100 ml of water; 1-cm light path. These solutions were contained in a special tandem cell with quartz windows which was built in our laboratory. This chemical filter transmits uv radiation with high efficiency in the spectral region 250–295 nm but absorbs the light in the 300–400-nm region. Use of excitation light of narrower band width, though sometimes desirable, was not practical due to loss in light intensity and concomitant increase in noise in the detected signals.

The wavelength of the fluorescent light was selected with a Jarell Ash double monochromator (Model 82-410) at a spectral resolution of 15 nm. Excitation light was removed by a cut-off filter composed of 0.1 M sodium biphthalate in water, 1-cm light path, which effectively absorbs all light below 300 nm. An elasto-optical light modulator (Morvue Model PEM-3) is used to modulate selectively the circularly polarized component of the fluorescence. The original polarizer which is attached to the exit side of the modulator was replaced by a polarizer which is suitable for uv light (Polacoat, formula UV-105). The CPL instrument was calibrated as described previously (Steinberg and Gafni, 1972). The circular polarization of luminescence is expressed by the emission anisotropy factor, g_{em}, defined as $g_{em} = 2\Delta f/f$, where Δf is the intensity of the circularly polarized part of the fluorescence, defined positive for left-handed circular polarization, and f is the total intensity of the fluorescence light. The absorption anisotropy factor, g_{ab}, is defined in an analogous manner to the emission anisotropy factor and is given by $g_{ab} = 2(\epsilon_l - \epsilon_r)/(\epsilon_l + \epsilon_r) = \Delta\epsilon/\epsilon$ (Kuhn, 1958), where ϵ_l and ϵ_r are the molar extinction coefficients for left-handed and right-handed circularly polarized light, respectively, and ϵ is the average molar extinction coefficient.

Absorption spectra were measured on a Zeiss Model PMQ II spectrophotometer. Circular dichroism spectra were measured on a Cary 60 spectropolarimeter equipped with a 6002 CD accessory. Corrected fluorescence spectra were obtained on a Turner 210 spectrofluorimeter or on a Hitachi-Perkin-Elmer fluorimeter (Model MPF-3). For solutions with optical density higher than 0.1/1-cm optical path, a special cell of 1.5-mm light path (Aminco) was used.

Results

Subtilisin Carlsberg-$PhCH_2SO_2$. The absorption and emission spectra, as well as the spectral behavior of the absorption and emission anisotropy factors, g_{ab} and g_{em}, respectively, of subtilisin Carlsberg-$PhCH_2SO_2$, are presented in Figure 1 for a variety of experimental conditions. The marked dependence of the emission spectrum on the excitation wavelength is noteworthy. On excitation at 280 nm the fluorescence exhibits a peak at 308 nm, with a shoulder at about 360 nm appearing at the higher pH studied (pH 8.3). In contrast, on excitation at 300 nm, the fluorescence peak appears in the range of 350–360 nm, shifting to the longer wavelength range with increase in pH. It may be recalled that this protein contains 13 tyrosine residues and only a single tryptophan residue. Thus, excitation light at 280 nm is predominantly absorbed by the tyrosine residues, which emit in the shorter wavelength region near 308 nm. On excitation at 300 nm, light absorption by the tyrosine residues is low, and the tryptophan emission predominates. This is clearly demonstrated by the excitation spectra for light emitted at 310 and 400 nm (see Figure 2), the species emitting at these two wavelengths obviously having significantly different absorption spectra.

Inspection of the fluorescence spectrum excited at 280 nm (see Figure 1) shows that the quantum yield of the Trp is enhanced relative to that of Tyr on raising the pH from 5.0 to 8.3. This point is not obvious from the spectrum excited at 300 nm presented in the same figure, since all emission spectra have been normalized to equal light intensity at their peaks in order to better detect the pH dependent λ_{max}. The shape of the emission spectrum excited at 280 nm does not depend on pH in the spectral range below 335 nm, indicating that Trp emission in this range is negligible and that Tyr emission probably predominates well above 335 nm. At wavelengths longer than where the shoulder appears, *i.e.*, above 360 nm, the fluorescence is probably due mostly or entirely to Trp emission. In view of the fact that there are 13 tyrosines present, it is quite possible that even upon excitation at 300 nm the emission contains some contribution from Tyr emission. The shift in the emission spectrum upon varying the pH may thus be due to the change in relative quantum yields of the Trp and Tyr residues rather than to a

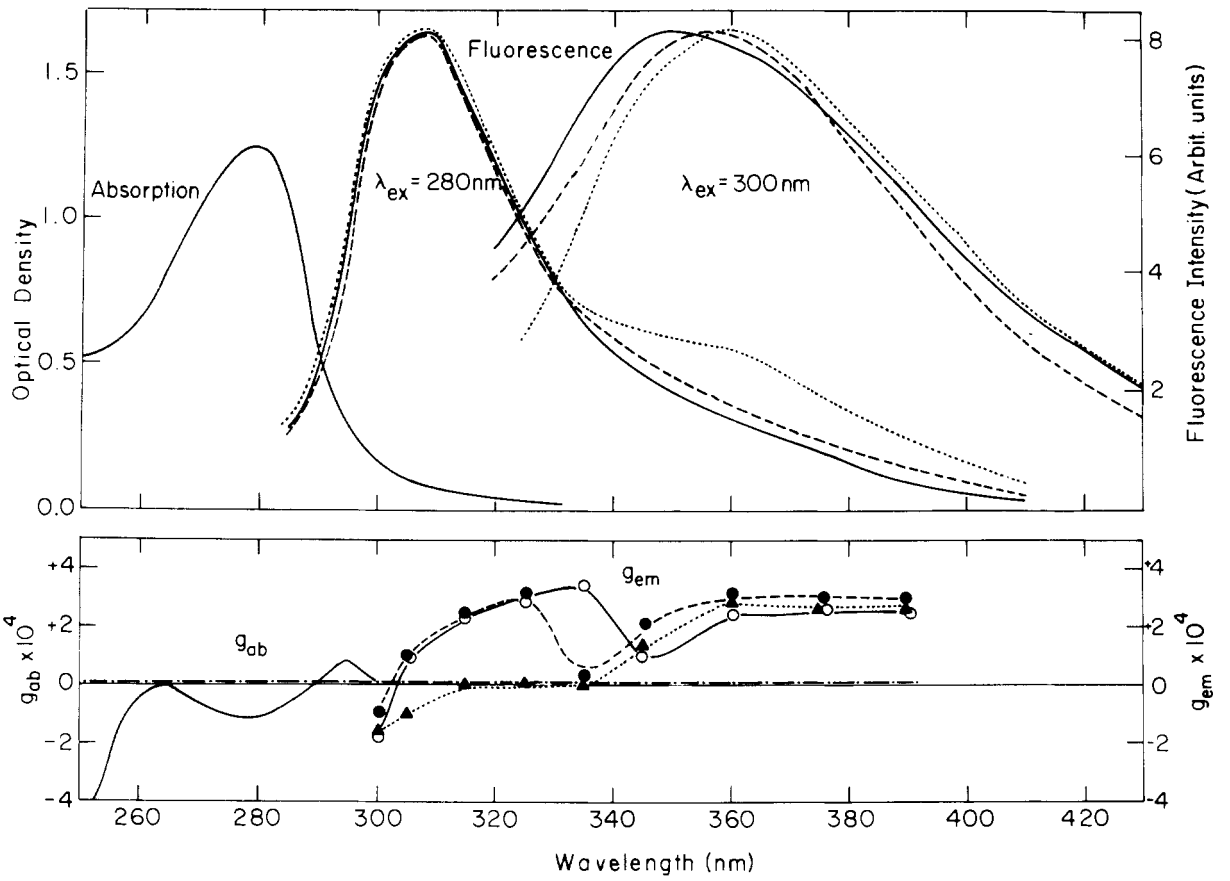

FIGURE 1: Spectroscopic data for subtilisin Carlsberg-$PhCH_2SO_2$. Absorption, 1.3 mg/ml of protein in 0.1 M phosphate buffer (pH 6.8). Absorption anisotropy factor, g_{ab}, 1.3 mg/ml of protein in 0.1 M phosphate buffer (pH 6.8) (—) and in 8 M guanidine hydrochloride (- - - -). Fluorescence spectra, 0.12 mg/ml of protein in 0.1 M acetate buffer (pH 5) (—); in 0.1 M phosphate buffer (pH 6.8) (- - -); and in 0.1 M Tris buffer (pH 8.3) (···). The excitation wavelengths were 280 or 300 nm with band pass of 10 nm, as indicated. The resolution of the fluorescence spectra is 2.5 nm. The various fluorescence spectra were normalized to equal height at their respective peaks, *i.e.*, for excitation at 280 nm at the tyrosine peak at 308 nm and for excitation at 300 nm at the tryptophan peak at 350–360 nm. Emission anisotropy factor, g_{em}, protein 1.5 mg/ml in 0.1 M acetate buffer (pH 5) (▲); in 0.1 M phosphate buffer (pH 6.8) (○); in 0.1 M Tris buffer (pH 8.3) (●); and in 8 M guanidine hydrochloride (- - - -). The excitation light for the CPL spectra was filtered by a broad band chemical filter (see Materials and Methods). Temperature, ~22°.

change in the emission spectrum of the single Trp residue in the protein.

Let us now turn to the spectra of the absorption and emission anisotropy factors. The magnitude of g_{ab} varies across the spectral range studied, which is only to be expected in view of the large number of chromophores involved (13 Tyr + 1 Trp). It is, however, hopeless to assign to any specific chromophore its contribution to g_{ab} at any point in the spectrum in view of the extensive overlap of the spectra of the various chromophores involved. On the whole, the absolute magnitude of g_{ab} is smaller than g_{em}, which is possibly due to more extensive cancellation of contributions of opposite sign in the former case. The CD spectrum does not show a strong dependence on pH (James, Chen, and Roche, to be published).

In marked contrast to the spectrum of g_{ab}, much more definitive and interesting conclusions can be drawn from the spectrum of g_{em}. It should first be noted that due to experimental limitations the excitation in this case is performed by light with a broad spectral range (see Materials and Methods). The excitation light in this case is, however, as effective as light of 280 nm in the relative excitation of Tyr and Trp residues, since the filter used in the excitation beam passes predominantly the 275-nm band of the high-pressure mercury arc. The spectrum of g_{em} shown in Figure 1 is thus related to the emission spectrum shown in the same figure for excitation by light of 280 nm. Therefore, g_{em} below 335 nm characterizes the fluorescence of Tyr residues, while g_{em} above 360 nm predominantly characterizes the fluorescence of the single Trp residue in the molecule. Indeed, in the wavelength region above 360 nm g_{em} assumes a constant value, as expected for a homogeneous population of chromophores. Such behavior was found before for g_{em} of other proteins which contain a single Trp residue (Steinberg *et al.*, 1974). It may be noted that g_{em} assumes the same sign and magnitude at the three pH values studied (5.0, 6.8, and 8.3). The asymmetric perturbation, and hence the environment, of the Trp residue thus seems to be unchanged in this pH range. This result supports the suggestion proposed above that the shift in the emission spectrum of the Trp residue with pH observed upon excitation at 300 nm is due rather to an interplay between the relative intensities between the Tyr and Trp emission than to a genuine shift in the Trp emission which would imply a change in its environment.

An entirely different, and more varied, behavior is observed for g_{em} in the spectral range of 300–360 nm, due to the Tyr emission. At none of the pH's studied is the Tyr g_{em} constant, which no doubt reflects the fact that there are 13 Tyr residues which most probably possess different optical activities and somewhat different emission spectra. The dependence of the Tyr g_{em} on pH is rather interesting. It is quite pronounced and reflects definite changes in the environment of at least some of the Tyr residues. The change

taking place between pH 5.0 and 6.8 extends throughout the pertinent spectral range, many Tyr residues apparently being affected. A finer and more specific change in g_{em} is observed on changing the pH from 6.8 to 8.3; g_{em} is affected only above 325 nm, probably indicating that only a limited subgroup of the Tyr population experiences a change in environment in this process.

Both the CD and CPL of subtilisin Carlsberg-$PhCH_2SO_2$ vanish within experimental accuracy in 8 M guanidinium hydrochloride (see Figure 1). This behavior of subtilisin Carlsberg-$PhCH_2SO_2$ is similar to that found for many other proteins under similar denaturing conditions, the macromolecular conformation being disrupted to such an extent that asymmetric perturbations of the chromophores involved become undetectable (Steinberg *et al.*, 1974).

Subtilisin Novo-$PhCH_2SO_2$. The absorption and emission spectra, as well as the spectral behavior of g_{ab} and g_{em}, of subtilisin Novo-$PhCH_2SO_2$ are presented in Figure 3. As can be seen from this figure, the emission spectrum depends markedly on the excitation wavelength, though to a less dramatic extent than in the case of subtilisin Carlsberg-$PhCH_2SO_2$. On excitation at 280 nm the fluorescence peak appears at about 337 nm and is no doubt due to the three tryptophan residues in the molecule. A pronounced shoulder appears, however, in the fluorescence spectrum below 320 nm and may be attributed to the emission of the ten tyrosine residues in the molecule. The fluorescence spectrum obtained upon excitation at 300 nm, where the Trp residues are preferentially excited, supports this assignment. The emission peak at 337 nm is affected only to a minor extent, while the shoulder below 320 nm disappears. The excitation spectrum for light emitted from subtilisin Novo-$PhCH_2SO_2$ at 310 and 400 nm is shown in Figure 2. It may be noted that while the two excitation spectra are distinct, their separation is appreciably smaller than in the case of subtilisin Carlsberg-$PhCH_2SO_2$. This is probably due to appreciable transfer of excitation energy from Tyr to Trp residues in subtilisin Novo-$PhCH_2SO_2$; the excitation spectrum for light emitted at 400 nm thus indicates light absorption by Tyr residues with a resulting shift of the spectrum to the blue relative to a pure Trp absorption spectrum. It is of interest to note that the emission spectra above 340 nm are very similar when excited at 280 and 300 nm and thus do not seem to contain any contribution from Tyr emission. At 330 nm the Tyr contribution is just a few per cent of the total emission at this wavelength. The tyrosine contribution to the fluorescence starts to be dominant below 315 nm.

As in the case of subtilisin Carlsberg-$PhCH_2SO_2$, the CD spectrum of subtilisin Novo-$PhCH_2SO_2$ shown in Figure 3 is difficult to resolve into contributions from the different chromophores involved. The CPL spectrum is much more informative. Obviously, only Tyr and Trp residues are involved, the former dominating the region below 315 nm and the latter the region above 330 nm. As explained above, the CPL spectrum was obtained under conditions in which both tyrosine and tryptophan residues are excited. In marked contrast to the case of subtilisin Carlsberg-$PhCH_2SO_2$, the g_{em} attributable to Trp emission of subtilisin Novo-$PhCH_2SO_2$, in the spectral range above 330 nm, is highly variable, even changing sign at 345–350 nm. It may thus be concluded that the three tryptophan residues in subtilisin Novo have different optical activity and different emission spectra. Moreover, the environment of the one or two tryptophan residues which show positive optical activity seems

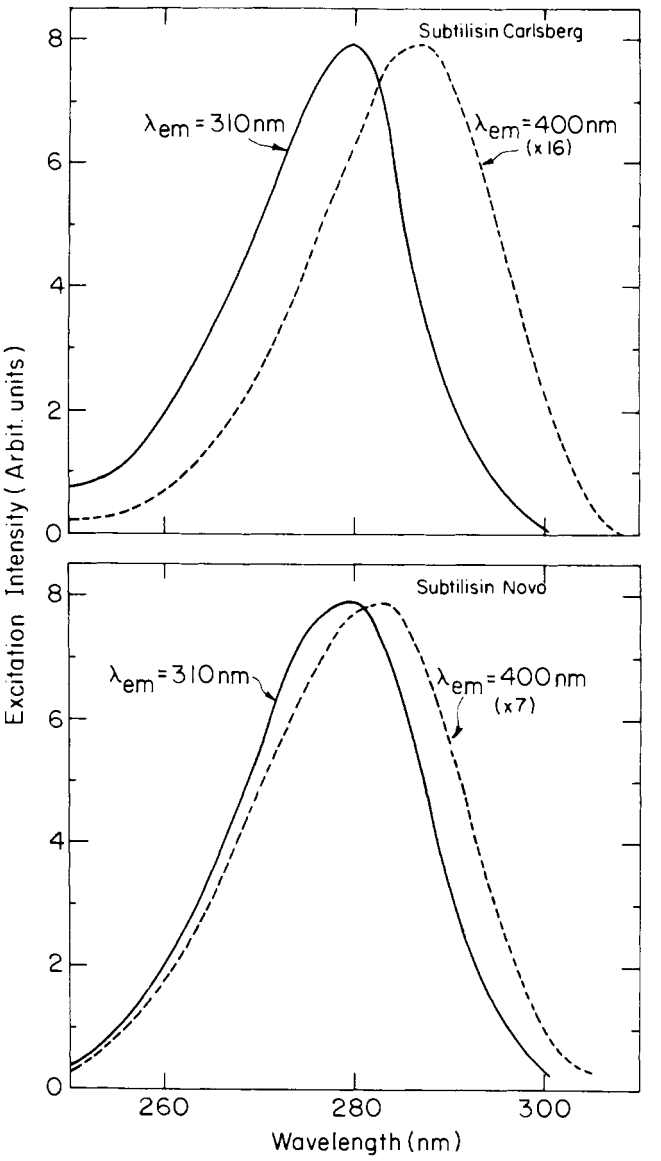

FIGURE 2: Excitation spectra of subtilisin Carlsberg-$PhCH_2SO_2$ (0.12 mg/ml) and subtilisin Novo-$PhCH_2SO_2$ (0.1 mg/ml) in 0.1 M phosphate buffer (pH 6.8). Emission wavelengths are 310 nm, at which tyrosine emission dominates; and 400 nm, at which tryptophan emission dominates. The resolution of the excitation and emission monochromators was 6 and 10 nm, respectively. Temperature, ~22°.

to vary detectably with pH, since g_{em} in the range of 330–345 nm is significantly pH dependent. The heterogeneity of the tyrosine residues with respect to optical activity and emission spectra is disclosed by the marked variation of g_{em} in the region 300–315 nm. Also in this case the asymmetric perturbations due to the environment of at least some of the Tyr residues seems to change with pH, especially in the pH range 5.0–6.8.

As seen in Figure 3, both CD and CPL vanish within the limits of detection in 8 M guanidinium hydrochloride, a behavior common to all other proteins studied which contain no internal cross-links (Steinberg *et al.*, 1974).

Fluorescence and CPL of Other Subtilisin Novo Derivatives. It has been shown that subtilisin Novo can be chemically modified by conversion of the active site Ser^{221} into a thiol derivative (Polgar and Bender, 1966; Neet and Koshland, 1966) which shows hydrolase activity and is structurally identical with the native enzyme (Kraut *et al.*, 1969) except for the replacement of the oxygen atom of Ser^{221} by

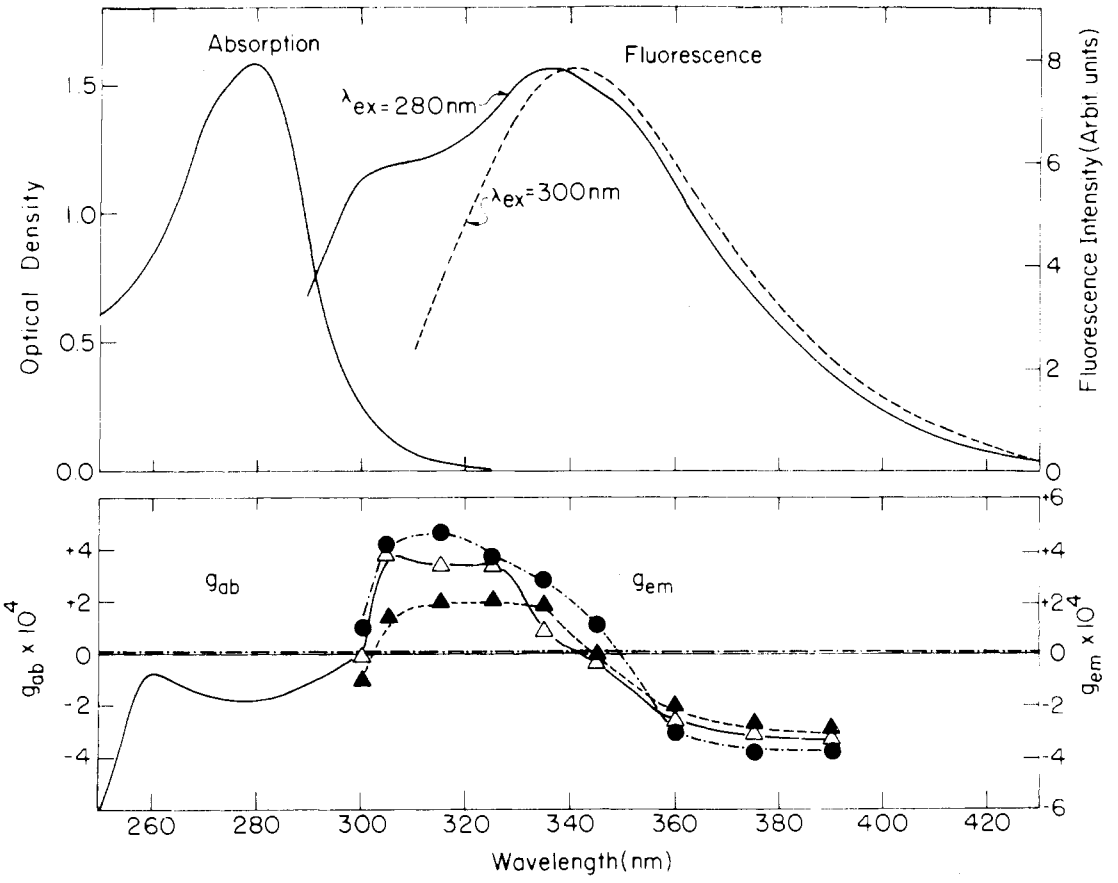

FIGURE 3: Spectroscopic data for subtilisin Novo-$PhCH_2SO_2$. Absorption, 1.45 mg/ml of protein in 0.1 M phosphate buffer (pH 6.8). Absorption anisotropy factor, g_{ab}, 1.45 mg/ml of protein in 0.1 M phosphate buffer (pH 6.8) (—) and in 8 M guanidine hydrochloride (- - - -). Fluorescence spectra, 0.1 mg/ml of protein in 0.1 M phosphate buffer (pH 6.8). The excitation wavelengths are 280 or 300 nm as indicated, with a spectral resolution of 10 nm. The spectral resolution of the fluorescence is 2.5 nm. Emission anisotropy factor, g_{em}, 1.5 mg/ml of protein in 0.1 M acetate buffer (pH 5) (▲); in 0.1 M phosphate buffer (pH 6.8) (△); in 0.1 M Tris buffer (pH 8.3) (●); and in 8 M guanidine hydrochloride (- - - -). The excitation light for the CPL spectra was filtered through a broad band chemical filter (see Materials and Methods). Temperature, ~22°.

a sulfur atom. The synthetic enzyme thus produced is not autolytic. Since its fluorescence properties are identical with those of native subtilisin Novo (Neet *et al.*, 1968) thiolsubtilisin-Novo is a good substitute for subtilisin Novo for physical measurements where autolysis may be troublesome. In order to explore the effects of active-site directed reagents on the conformation of the protein, which can be reasonably assumed to mimic the binding of substrates and the formation of enzyme–substrate complexes, we have examined in a comparative manner the CPL of thiolsubtilisin-Novo, subtilisin Novo-$PhCH_2SO_2$, and subtilisin Novo-nisyl. The relevant spectroscopic data are summarized in Figure 4. The fluorescence spectra indicate that, relative to $PhCH_2SO_2$, nisyl quenches the tryptophan fluorescence. The difference in molar absorbance between the two derivatives is small and therefore the observed quenching is not due to an inner filter effect. The spectra of g_{em} are obviously different for the three derivatives of subtilisin Novo, and demonstrate that introduction of the $PhCH_2SO_2$ or nisyl groups at the active site of this enzyme produces significant and different conformational changes in the environment of at least a few of the tyrosine and tryptophan residues in the protein molecule.

Discussion

Longworth (1971, 1972) first noted that the subtilisins are exceptions to the general rule that, in proteins containing both tyrosine and tryptophan residues, low quantum yield of tyrosine residues and efficient transfer of electronic excitation energy from tyrosine to tryptophan lead to a predominance of tryptophan emission even when the fluorescence is excited in the absorption band of tyrosine. This observation of pronounced Tyr emission in the subtilisins has been discussed in terms of the available energy transfer routes calculated on the basis of the X-ray crystallographic coordinates which have been published for subtilisin Novo (R. S. Roche and M. Shinitzky, in preparation) and is confirmed by our results. The emission and excitation spectra for subtilisin Carlsberg-$PhCH_2SO_2$ and subtilisin Novo-$PhCH_2SO_2$ (Figures 1–3) demonstrate rather strikingly the separation between tyrosine and tryptophan emission spectra in these molecules. The separation between the excitation spectra for light emitted at 310 and 400 nm is not complete and it is seen that some energy transfer from tyrosine to tryptophan does in fact occur (Longworth, 1972) though, clearly, it is not completely efficient. It is also seen in Figure 2 that the separation of tyrosine and tryptophan emissions in subtilisin Carlsberg-$PhCH_2SO_2$ is more pronounced than in subtilisin Novo-$PhCH_2SO_2$, which indicates that energy transfer in the subtilisin Carlsberg-$PhCH_2SO_2$ molecule is less efficient than it is in subtilisin Novo-$PhCH_2SO_2$. The explanation for this observation most probably lies in the fact that in subtilisin Carlsberg there is only one tryptophan (Trp-113) whereas in subtilisin Novo there are three (Trp-106, -113, and -241), hence more possibilities for energy transfer (R. S. Roche and M. Shin-

itzky, in preparation). We have found that the separations seen in the excitation spectra shown in Figure 2 are only slightly sensitive to pH in the range of 5.0–8.3. From this we can conclude that the energy transfer routes are not perturbed by changes in pH and hence the molecules do not undergo gross conformational changes in this pH range. This observation is consistent with the fact that the CD spectra of subtilisin Novo-$PhCH_2SO_2$ and subtilisin Carlsberg-$PhCH_2SO_2$ are not very sensitive to pH, both in the peptide and aromatic regions (James, Chen, and Roche, to be published).

For a specific chromophore, g_{ab} at the longest wavelength absorption band and g_{em} should assume equal sign and magnitude if the conformation and environment of the chromophore do not change upon electronic excitation (Steinberg, 1974; Steinberg *et al.*, 1974). A comparison between g_{ab} and g_{em} is, however, not feasible in the protein derivatives studied, since only a small number of the chromophores contributing to the CD are represented in the CPL. Only Tyr and Trp residues show up in the CPL, and even then not all of these residues are necessarily fluorescent (Yashinsky, 1972). Furthermore, the different Tyr and Trp residues do not have the same quantum yield, and their relative contributions to the CPL and CD are therefore different. All of these factors probably account for the observation that the CD of subtilisin Carlsberg-$PhCH_2SO_2$ and subtilisin Novo-$PhCH_2SO_2$ are not very sensitive to pH in the range studied (5.0–8.3) (James, Chen, and Roche, to be published) while the CPL shows pronounced variation with pH. The gross molecular conformation, as reflected by the multitude of chromophores involved in the CD, is apparently not sensitive to pH, while local changes around some of the chromophores contributing to the CPL, which are much fewer in number, do occur. In this context, a discussion of the CPL results obtained for the various derivatives of subtilisin Novo and the available structural X-ray data is appropriate. Only minor differences were found at 2.5-Å resolution between the structures determined in the crystals of space group C_2 obtained at pH 5.9 and those of space group $P2_1$, crystallized at pH 9.1 (Drenth *et al.*, 1971). As shown in Figure 3, the CPL spectrum of subtilisin Novo-$PhCH_2SO_2$ changes markedly with pH in the range 5.0–8.3. As concluded above, the changes in CPL reflect local changes around the fluorescing chromophores, and these seem to be smaller than the limit of resolution of the available X-ray results. Similarly, electron density difference maps at 2.5-Å resolution between subtilisin Novo and subtilisin Novo-$PhCH_2SO_2$ (Wright *et al.*, 1969) show only perturbations in the region of His^{64}, which moves 4.0 Å on formation of the derivative, and Met^{222}. The pronounced difference in the CPL spectra of thiolsubtilisin-Novo and subtilisin Novo-$PhCH_2SO_2$ shown in Figure 4 thus reflects changes in the vicinity of the fluorescing chromophores which the X-ray data at the available resolution cannot disclose.

In previous studies of proteins which contain a single tryptophan residue it was found that g_{ab} at the red edge of the absorption band was comparable in magnitude and equal in sign to g_{em} (Steinberg *et al.*, 1974) (human serum albumin is an exception due to the heterogeneous population of molecules that make up this protein). Though it is very difficult to resolve the CD spectrum of subtilisin Carlsberg-$PhCH_2SO_2$, it is tempting to assign the positive peak in the spectrum of g_{ab} at 295 nm to the single Trp residue in the molecule in view of the positive value of the g_{em}

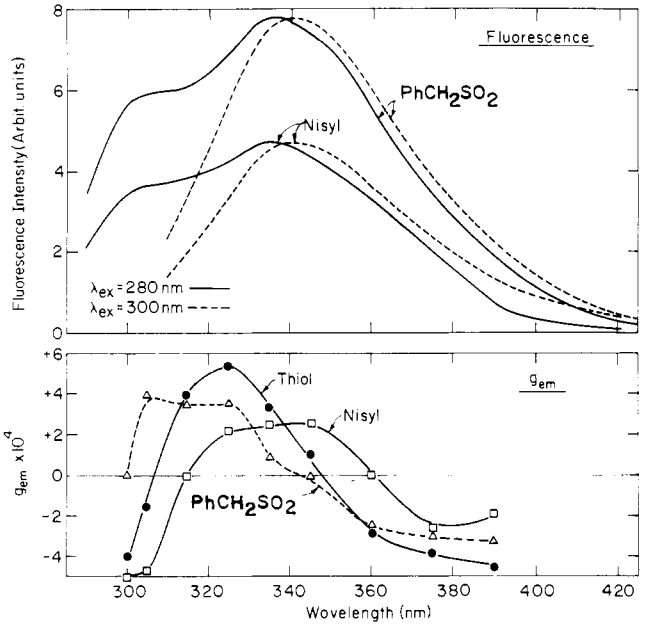

FIGURE 4: Fluorescence and CPL spectra of subtilisin Novo derivatives. Fluorescence, 0.1 mg/ml of subtilisin Novo-$PhCH_2SO_2$ in 0.1 M phosphate buffer (pH 6.8), and 0.1 mg/ml of subtilisin Novo-nisyl in the same buffer. The excitation wavelengths are 280 or 300 nm, as indicated, with spectral resolution of 10 nm. The resolution of the fluorescence spectra is 6 nm. Emission anisotropy factor, g_{em}: subtilisin Novo-$PhCH_2SO_2$ (△), subtilisin Novo-nisyl (□), and thiolsubtilisin Novo (●) in 0.1 M phosphate buffer (pH 6.8). Protein concentration, 1.2 mg/ml. Temperature, ~22°.

of this Trp residue (see Figure 1). It may be recalled that one or two of the tryptophan residues in subtilisin Novo-$PhCH_2SO_2$ exhibit a positive g_{em}. *If* conformational homology exists between subtilisin Carlsberg and subtilisin Novo, it is again tempting to assume that Trp-113 is involved in the positive g_{em} of S. Novo in the range 330–345 nm, in analogy to Trp-113 of subtilisin Carlsberg.

The CPL of subtilisin Carlsberg-$PhCH_2SO_2$ clearly demonstrates that there are changes in the environment of at least some of the tyrosine residues in the pH range 5.0–8.3. Between pH 6.8 and 8.3 the changes seem to be more restricted to those Tyr residues which emit predominantly around 335 nm. Similarly, changes in the environment of both the fluorescent Tyr and Trp residues is indicated in subtilisin Novo-$PhCH_2SO_2$ on changing the pH from 5.0 to 8.3. Thus, variations in the enzymatic activity of these proteins in the above pH range are not necessarily due exclusively to ionization of side chains; conformational changes, though local, may play an important role in this respect.

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Iron-Donating Properties of Transferrin†

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ABSTRACT: The transferrin molecule has two specific metal-binding sites, each of which may provide iron for the biosynthesis of hemoglobin by reticulocytes. Diferric human transferrin was shown to be a better iron donor, per iron atom, for rabbit reticulocytes, than was monoferric transferrin obtained by isoelectric focussing. The difference in binding of ^{125}I-labeled monoferric and diferric transferrin to reticulocytes may be sufficient to account for the difference in iron uptake. In contrast, diferric and monoferric rabbit transferrin both donated iron to reticulocytes at the same rate, per iron atom. In an experiment using ^{55}Fe/^{59}Fe doubly labeled transferrin, one iron binding site of human transferrin was a better iron donor than the other. In rabbit transferrin, the two sites appeared to function equivalently. Care was taken in these experiments to demonstrate that labeled iron added to dilute solutions of transferrin was indeed specifically bound to the protein. A liquid scintillation counting procedure, simpler than existing methods, was developed to quantitate ^{55}Fe and ^{59}Fe in blood.

The major physiologic role of the plasma iron-transport protein, transferrin, is the delivery of iron to hemoglobin-synthesizing immature red blood cells. This glycoprotein molecule consists of a single polypeptide chain of molecular weight near 80,000 (Greene and Feeney, 1968; Mann *et al.*, 1970) on which are disposed two specific iron-binding sites which are very similar, if not identical, by a variety of thermodynamic and spectroscopic criteria (Aasa *et al.*, 1963; Aisen *et al.*, 1966; Binford and Foster, 1974). Fletcher and Huehns (1967; Fletcher, 1969) have presented evidence which suggests that the two sites are functionally heterogeneous; one site appears to be a better iron donor for reticulocytes than the other. They also observed that iron-saturated transferrin is a better source of iron for reticulocytes than partially saturated transferrin. A similar saturation effect was observed in the delivery of iron to the liver (Fletcher, 1971) and a reverse effect was postulated to be important in iron transport to the placenta (Fletcher and Huehns, 1968). Other studies failed to confirm these findings and a myriad of conflicting reports concerning these effects now exists (Lane, 1973; Hahn, 1973, 1974; Ganzoni *et al.*, 1972; Chernelch and Brown, 1970; Lane and Finch, 1970;

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