The Contribution of the Single Tryptophyl Residues to the Fluorescence Emission of Porcine Elastase

GIULIO JORI and GUIDO GALIAZZO
Istituto di Chimica Organica, Università di Padova, Italy

(Z. Naturforsch. 29b, 201-205 [1974]; received November 26, 1973)

Tryptophan emission, specific photooxidation, D2O effect

In order to evaluate the contribution of the single tryptophyl residues to the fluorescence emission of elastase, the latter was analyzed as a function of pH, as well as in the presence of some perturbants and quenchers. Moreover, specific tryptophyl residues were converted to formyl-kynurenine by photosensitized oxidation. Our results suggest that the two external tryptophans 26 and 164 account for at least 50% of the total emission; out of the two partially buried tryptophans, the residue 83 is practically non-fluorescent, whereas the residue 12 contributes about one fourth of the elastase fluorescence. The residual fluorescence (about 25% of the total emission) is to be ascribed to the three deeply buried tryptophans 39, 132 and 232.

Introduction

The study of the intrinsic fluorescence emission of proteins is an increasingly active area of research. Besides its obvious relevance for the mechanism of the photobiological processes involving proteins, the fluorescence emission of proteins can provide significant information on several ground state properties of these compounds, such as their overall conformation, their stability to denaturants, the mechanism of interaction with substrates, and the intramolecular distances among specific side chains (see, for a recent review, ref. 1). The main difficulty for a detailed interpretation of the fluorescence behaviour of proteins is given by the multiplicity of potential emitters (in general, the tyrosyl and tryptophyl side chains2), as well as by the high sensitivity of the fluorogenic residues to the nature of their molecular microenvironment1-3.

Clearly, more research is needed for elucidating the influence of the various conformational parameters on the efficiency of the radiative deactivation of the excited singlet state of the aromatic residues. Toward this aim, one can take advantage of the increasing number of protein structures which have been resolved at an atomic detail by X-ray crystallography. Actually, on this basis, LEHRER and FASMAN3 and WEINRYB and STEINER4 succeeded in assessing the relative contribution of several aromatic fluorophors of the total emission of lysozyme and, respectively, papain. A comprehensive treatment of the influence of intramolecular interactions in proteins on the fluorescence parameters of the tryptophyl residues has been published recently4.

In this paper, we report our findings on the fluorescence of the tryptophyl residues of elastase. The three-dimensional structure of this protein has been revolved5-7 and found to be very similar with that of other serine proteases, such as trypsin and a-chymotrypsin. Moreover, recent studies concerning the photosensitized oxidation of elastase8 demonstrated the possibility of performing the stepwise selective modification of several tryptophyl residues. Finally, a preliminary study in our laboratory9 pointed out that the fluorescence emission of elastase is sensitive to the conformation of the protein molecule.

Experimental

Porcine pancreatic elastase ( Worthington Biochemical Corp., twice crystallized) was purified by ion-exchange column chromatography9. The elastase derivatives modified at specific tryptophyl residues were obtained by proflavine-sensitized photooxidation under controlled experimental conditions9; their purity was checked by chromato-
graphic and amino acid analysis. Guanidinium chloride (Mann BioResearch Co., ultrapure) was crystallized once from ethanol. All other chemicals were commercial products of the highest available grade.

The fluorescence spectra were recorded by an Hitachi Perkin Elmer Model MPF 2A spectrophotofluorometer, equipped with a Philips R 106 photomultiplier tube. The procedures adopted for recording and correcting the spectra were described previously\textsuperscript{10-11}. Small but significant corrections were also made from a solvent and buffer blank. In all cases, the optical density of the protein solutions was about 0.13 at the excitation wavelength. The absorption measurements were obtained with a Cary model 15 spectrophotometer, which was operated at 25 °C.

### Results

**Fluorescence properties of the native protein**

All the fluorescence measurements were performed using 295 nm as the excitation wavelength; this is located in the red edge of the tryptophan absorption spectrum, where the high repolarization of the protein fluorescence emission demonstrates the absence of any significant intertryptophyl energy transfer\textsuperscript{12}; therefore, the observed emission can be regarded as the sum of the contributions of the single tryptophyl fluorophors. The fluorescence parameters, which were shown\textsuperscript{5} to be mainly dependent on the nature of the surroundings of the tryptophyl side chains in a protein molecule (i.e., the position of the emission maximum, the emission quantum yield and the spectral band width), were measured for elastase in aqueous solutions at pH 6.3, 3.5 and 2.5; previous investigations\textsuperscript{8} showed that, at these pH values, the protein exists in its native conformation, in a partially loosened conformation, and in an extensively denatured conformation, respectively.

As shown in Table I, lowering the pH over the aforesaid range brought about no important change of the examined properties, except for a depression of the fluorescence quantum yield at pH 2.5. On the other hand, the presence of D\textsubscript{2}O or dioxane had drastic effects, especially on the emission quantum yield: whereas in the former solvent, the yield of the radiative decay from the excited singlet state was almost doubled, the addition of dioxane caused a strong quenching of the emission, the effect being complete at relatively low levels of the organic solvent. The hypsochromic shift of the emission maximum in aqueous dioxane solutions is probably indicative of a somewhat less polar environment for the tryptophyl residues\textsuperscript{1-5}. As shown in Fig. 1, the presence of 10% dioxane induced only small perturbations of the absorption spectrum of elastase in the near UV-region, where the absorption is dominated by tryptophan. On the other hand, the spectrum obtained in the presence of 40% dioxane clearly shows that, under these conditions, the structure of the protein molecule is largely disorganized.

The addition of nitrate, a known quencher of protein fluorescence, also resulted in a decrease of the emission according to the plot shown in Fig. 2.

<table>
<thead>
<tr>
<th>Medium</th>
<th>( \lambda_{\text{max}} ) (nm)</th>
<th>( \Delta \lambda ) (nm)</th>
<th>( q )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water, pH 6.3</td>
<td>338</td>
<td>56</td>
<td>0.22</td>
</tr>
<tr>
<td>Water, pH 3.5</td>
<td>336</td>
<td>58</td>
<td>0.19</td>
</tr>
<tr>
<td>Water, pH 2.5</td>
<td>341</td>
<td>56</td>
<td>0.13</td>
</tr>
<tr>
<td>99.7% D\textsubscript{2}O, pD 6.2</td>
<td>339</td>
<td>56</td>
<td>0.38</td>
</tr>
<tr>
<td>10% Dioxane, pH 6.1</td>
<td>332</td>
<td>52</td>
<td>0.12</td>
</tr>
<tr>
<td>40% Dioxane, pH 6.2</td>
<td>330</td>
<td>50</td>
<td>0.09</td>
</tr>
</tbody>
</table>

\( a \) Spectral band width at half height.

\( b \) Total emission quantum yield; the estimated error is ±0.02.
Fig. 2. The effect of NaNO₃ concentration on the fluorescence quantum yield of elastase. All measurements were performed at 23 °C ± 1 °C in aqueous solutions at pH 6.3. F₀ = fluorescence quantum yield of elastase in the absence of added NaNO₃.

The Stern-Volmer plot indicates a residual fluorescence (approx. 25%) apparently arising from one or more indole moieties inaccessible to the nitrate ions. The quenching efficiency was unchanged by the addition of sodium chloride up to about 3 M, which allows one to rule out any effect of charge interactions.

**Fluorescence properties of photooxidized derivatives of elastase**

The importance of some tryptophyl residues for the fluorescence emission of elastase was also investigated by using elastase derivatives photooxidized at specific tryptophans. Control experiments demonstrated that the photooxidation product of tryptophan, *i.e.* formyl-kynurenine, has no appreciable emission below 400 nm, when excited at 295 nm. Therefore, the observed fluorescence for each sample can be ascribed to the residual unmodified tryptophyl side chains. Moreover, kynurenyl derivatives display a minimum absorption at 295 nm, so that any filter effect on the absorption of the exciting radiation by tryptophan can be neglected.

The values obtained for the wavelength of maximum emission and the emission quantum yield of the various samples are summarized in Table II. Interestingly, the remarkable D₂O effect, which had been noticed for native elastase, almost completely disappears for the sample modified at tryptophan-26 and -164, unless the latter is exposed at relatively high denaturant concentrations.

**Discussion**

Previous investigations from this laboratory pointed out that, in native elastase, tryptophan-26 and -164 are essentially exposed at the surface of the molecule, whereas tryptophan-12 and tryptophan-83 become fully accessible to the aqueous solvent only upon lowering the pH of the medium to 2.5 and, respectively, 3.3. These conclusions are in reasonable agreement with those suggested by the X-ray structure of the protein. On these bases the data above described provide a sufficiently clear interpretation of the relative importance of several out of the seven tryptophyl residues for the process of fluorescence emission from elastase.

In the first place, our findings suggest that the two external tryptophyl side chains (i.e. tryptophan-26 and -164) play a major role in determining the emission quantum yield. In this connection, the most striking evidence is provided by the drastic drop of the emission quantum yield (about 50%) observed after conversion of the two aforesaid tryptophans to formyl-kynurenine. Actually, the comparative analysis of several conformation-dependent parameters pointed out that native elastase and its di-formyl-kynurenyl derivative possess a practically identical three-dimensional organization; the same point is stressed by the coincidence of the wavelength of maximum emission for the two compounds (see Tables I and II). Consequently, the observed decrease in the quantum yield cannot be the consequence of a different interaction of the residual indole fluorophores with either the solvent or their molecular microenvironment after photooxidation. The possibility remains that the de-

<table>
<thead>
<tr>
<th>Sample</th>
<th>Modified tryptophans</th>
<th>λ_{max}(nm)</th>
<th>q</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Trp-26, -164</td>
<td>337</td>
<td>0.12</td>
</tr>
<tr>
<td>1</td>
<td>Id. in 99.7% D₂O</td>
<td>337</td>
<td>0.15</td>
</tr>
<tr>
<td>1</td>
<td>Id. in 99.7% D₂O + 5 M guanidinium chloride</td>
<td>349</td>
<td>0.20</td>
</tr>
<tr>
<td>2</td>
<td>Trp-26, -164, -83</td>
<td>330</td>
<td>0.12</td>
</tr>
<tr>
<td>3</td>
<td>Trp-26, -164, -83, -12</td>
<td>351</td>
<td>0.05</td>
</tr>
</tbody>
</table>
pression of the intrinsic protein fluorescence is due to the oxidative destruction of two important emitters. This conclusion is strengthened by the noticeable fluorescence enhancement exhibited by native elastase in the presence of D\textsubscript{2}O. As known\textsuperscript{14}, the fluorescence emission of several indole derivatives, including tryptophan, is almost doubled when D\textsubscript{2}O is substituted for H\textsubscript{2}O; accordingly, the high sensitivity of the elastase fluorescence to the isotope effect must reflect the perturbation of the radiative decay of fluorescing indole moieties which are most readily accessible to the aqueous solvent. It may be recalled that the fluorescence emission of other proteins\textsuperscript{5,12}, in which the surface tryptophyl residues make a minor contribution to the overall quantum yield, is affected by heavy water to a very low extent. On the other hand, the elastase derivative devoid of the two external tryptophyl residues displayed no significant sensitivity to the presence of D\textsubscript{2}O, which demonstrates that these residues are the main responsible for the described phenomenon. Finally, the values found for the emission maximum and the spectral band width in the case of native elastase also suggest that the fluorescence of this protein is dominated by tryptophyl side chains located in a rather polar environment (see ref. 5).

The additional photooxidation of tryptophan-83 causes no appreciable further decrease of the fluorescence quantum yield of elastase. This fact immediately suggests that this residue is of minor importance for the emission process. Once again, previous conformational investigations demonstrated\textsuperscript{5} that this elastase derivative maintains a sufficiently tight spatial structure, although some limited rearrangements must occur (see, for example, the small blue shift of the fluorescence maximum). However, it is not clear from the present data which molecular factors are responsible for the quenching of the emission of tryptophan-83, since there was no significant variation of the fluorescence parameters even upon lowering the pH of the solution from 6.3 to 3.5; within this pH range, the molecular environment of tryptophan-83 must surely change, as demonstrated by its different accessibility to the photooxidizing agent\textsuperscript{5}.

On the contrary, tryptophan-12 appears to give a remarkable contribution to the elastase emission, since its conversion to formylkynurenine (see Table II) induces an approx. 50% decrease in the quantum yield of the residual fluorescence. Further, more, the experiments performed with unmodified elastase showed that the quantum yield undergoes some decrease within the pH interval 3.5-2.5, corresponding to a conformational transition of elastase such as to bring the originally buried tryptophan-12 into contact with the external solvent. The exact value of the contribution of tryptophan-12 cannot be precisely assessed from our data, since the elastase molecule modified at four tryptophyl residues exists in an extensively collapsed conformation: this is demonstrated both by previous studies\textsuperscript{9} and by the large bathochromic shift of the emission maximum, which approaches the one typical of L-tryptophan in aqueous solution. Therefore, the observed fluorescence decrease might be the consequence, at least in part, of a perturbation of the environment of the three remaining tryptophyl side chains. However, it can be observed that the quenching experiments with NaNO\textsubscript{3} indicate the presence of a non-quenchable fluorescence, corresponding to about 25% of the total emission. It appears reasonable to ascribe this fluorescence to the radiative deactivation of those indole emitters which are most deeply buried inside the elastase three-dimensional structure, and which cannot therefore be easily contacted by the added quencher. Significantly, the fluorescence quantum yield of the elastase derivative additionally modified at tryptophan-12 is also about 25% of that of native elastase. It is unlikely that such a correspondence is fortuitous; therefore, we tentatively assign the residual fluorescence emission to the three internal tryptophyl side chains, i.e. tryptophan-39, -132 and -232; tryptophan-12 alone should contribute about 25% of the elastase fluorescence. As regards these partially or deeply buried tryptophyl side chains, it is worthwhile noting that the absence of any influence of D\textsubscript{2}O on their fluorescence emission is probably a consequence of their location in hydrophobic regions of the protein molecule; actually, as shown in Table II, the isotope effect became quite evident as soon as the elastase molecule was unfolded by the addition of guanidinium chloride in order to destroy the ordered native geometry.

The same lines of evidence are provided by the fluorescence data obtained in the presence of dioxane, i.e. a known perturbant of the secondary and tertiary structure of proteins. In actual fact, as one can see from Table I, a major depression of the quantum yield is already apparent at relatively
low concentrations of dioxane; under these conditions, no gross conformational rearrangement of the elastase molecule has occurred, as clearly shown by the difference absorption spectrum. Therefore, any effect on the fluorescence emission must primarily involve the most accessible residues, which have the greatest probability to interact with the perturbant. On the contrary, only slight further changes of the fluorescence parameters were induced by increasing the concentration of dioxane to such values that the protein molecule undergoes a general denaturation rendering the various tryptophyl residues almost equally accessible to the solvent. In actual fact, in 40% dioxane, the indole fluorophores must be in a medium less polar than water: this is consistent with the reduction of the spectral band width and with the blue shift of the emission maximum.

In conclusion, our investigations suggest that, in elastase, the major contribution (approx. 50%) to the overall protein emission is given by the two most exposed tryptophans, i.e. tryptophan-26 and -164. Tryptophan-83 is unimportant for the process of radiative decay, whereas the side chain of tryptophan-12, by itself, should emit fluorescence with a significant quantum yield. The buried tryptophans-39, -132 and -232 must have a much lower emission quantum yield, corresponding in total to 25% of the whole elastase emission. These findings are rather uncommon, since in most proteins the indole moieties located in hydrophobic regions exhibit the greatest fluorescence quantum yield. However, Burstein et al. recently came to the conclusion that the specific quantum yield of tryptophyl residues in a polar environment should be higher than that of buried tryptophans. Now, several of the aforesaid aromatic residues of elastase are homologous with those of trypsin and α-chymotrypsin as regards the position in the primary sequence; the three proteins have closely similar tertiary structures, hence the environments of the various tryptophans should be very similar also. It would be of interest to extend the aforesaid approach to study the fluorescence emission of the other two proteases, in order to achieve a clearer understanding of the importance of the single molecular factors for enhancing or inhibiting the fluorescence emission of the tryptophyl residues in proteins.

3 S. S. Lehmann and G. Fasman, Biopolymers 2, 199 [1964].
4 I. Weinryb and R. F. Steiner, Biochemistry 9, 135 [1970].
14 L. Stryer, J. Amer. chem. Soc. 88, 5708 [1966].