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Exposure of tryptophanyl residues in α-lactalbumin and lysozyme

Quantitative determination by fluorescence quenching studies

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Summary. The effect of iodide ion on the tryptophyl fluorescence of the homologous proteins lysozyme and α-lactalbumin in their native form, as well as in their modified structures and in fragments from these proteins was studied. By assessing the contribution to the total fluorescence of the exposed and buried Trp residues, and of the respective fluorescence quantum yields, the quantization of the number of Trp exposed to the solvent for all the species studied was possible. Both native proteins show an important increase in the number of Trp residues exposed to the solvent when treated with denaturing agents. The peptides L-II (aa 13-105) and α-I (aa 1-90) from lysozyme and α-lactalbumin, respectively, showed Trp residues with different degree of exposure, whereas the smaller fragments, L-III (aa 106-129) and α-II (aa 91-123), had all their Trp residues exposed to the solvent.

Introduction

In spite of the fact that the degree of exposure of a specific amino acid residue in a protein can be recognized from X-ray crystallographic information, an interesting problem for the chemist is to find an appropriate way to describe the dynamic behaviour of proteins in solution. The Trp residues in native proteins can present different degrees of exposure to the solvent. It is known that the micro-environment of these residues (polarizability, microviscosity, availability of charged groups, possible specific interactions, etc.) will influence the fluorescence characteristics such as wave-length of maximum emission, bandwidth, quantum yield, etc. (Burstein et al. 1973; Cowgill 1967; Sun and Song 1977). In the case of proteins that present more than one Trp residue in their structure, the protein fluorescence will be conditioned by the addition of the fluorescent contributions of the individual Trp residues (Burstein et al. 1973).

Quenching studies of protein fluorescence by adding external molecules or ions, give information on the degree of exposure of Trp residues in

the macromolecule (Burstein et al. 1973; Eftink and Ghiron 1981; Omar and Schleich 1981). They have also been used to monitor structural changes in the protein. Molecular oxygen, acrylamide, and a number of ionic quenchers have been used quite extensively in studies with proteins. It has been demonstrated that oxygen and acrylamide have the capacity of penetrating at least in part to the interior of the protein, quenching the fluorescence of the well-known buried Trp residues (Calhoun et al. 1983a, b).

With the objective of studying the specific quenching of the Trp residues exposed in the homologous proteins lysozyme and α-lactalbumin, we employed a quencher of ionic type, which due to its charged nature does not penetrate to the interior of the protein matrix. The iodide ion was chosen due to its efficiency as a quencher and to the fact that its behaviour corresponds to that of a dynamic quencher, which allows the analysis of the results according to Lehrer (Eftink and Ghiron 1981; Lehrer 1971). Possible effects of the negative charge of the iodide ion were checked with a cationic quencher such as cesium ion, which has the disadvantage of having a very low efficiency (Eftink and Ghiron 1981).

In this work the quenching by iodide ion of the homologous proteins lysozyme and α-lactalbumin in their native form, as well as in their modified forms and in fragments resulting from CNBr-cleavage of these proteins is studied. Information about the degree of exposure of Trp residues in these species is obtained.

Materials and methods

Hen egg-white lysozyme grade I, bovine α-lactalbumin grade II, β-lactoglobulin from bovine milk, guanidine-HCl grade I, urea, sodium iodide, cesium chloride, HEPES and cyanogen bromide were obtained from Sigma Chem. Co.

α-Lactalbumin was purified by gel filtration on a column of Sephadex G-100 (2 × 80 cm) equilibrated and eluted with 0.15 *M* KCl. Reduction and carboxymethylation were carried out as described by Crestfield et al. (1963) for lysozyme, and by Brew et al. (1970) for α-lactalbumin.

CNBr-cleavage of Met-peptide bonds was carried out as described by Ferrer and Silva (1981).

Quenching measurements were made in eight samples of a given material with varying amounts of sodium iodide (0–0.8 *M*). They were prepared by diluting stock solutions of the model compound, of NaI and of NaCl, all prepared in buffer 2 m*M* HEPES pH 7.5. NaCl was used to keep the ionic strenght constant.

All solutions were freshly prepared, and a small amount of $S_2O_3^{2-}$ was added to the iodide stock solution to prevent I_3^- formation. The solutions were equilibrated at 25° C before and during the measurements.

Absorption spectra were recorded with a Varian Super Scan spectrophotometer and A_{295} was determined by direct reading.

Fluorescence spectra were recorded with a Perkin-Elmer 650-10S fluorescence spectrophotometer and the values of intensity of maximum emission

were determined by direct reading. An excitation wavelength of 295 nm was used in all cases to ensure selective excitation of the Trp residues.

Interpretation of data. If only one type of Trp residue accessible to the quencher is available, the quenching process follows the Stern Volmer relation:

$$\frac{I_0}{I} = 1 + K_{sv}[Q] \tag{1}$$

where I_0 and I are the fluorescence intensities in the absence and in the presence of quencher respectively, K_{sv} is the Stern-Volmer quenching constant and $[Q]$ is the quencher concentration.

By plotting I_0/I vs. $[Q]$, a straight line of slope higher than zero will be obtained. If only one type of Trp residues is available, and these are inaccessible to the quencher, the fluorescence I will be independent of the quencher concentration. In the presence of both accessible and inaccessible Trp residues, a plot of I_0/I against the Q concentration will show a downward curvature (Eftink and Ghiron 1981; Lehrer 1971). The Lehrer equation can be used to separate the fluorescence of the exposed and buried residues (Lehrer 1971).

$$\frac{I_0}{I_0 - I} = \frac{1}{F_w} + \frac{1}{F_w \cdot K_{sv}[Q]} \tag{2}$$

where Q, I_0, I and K_{sv} have the same meaning as in (1) and F_w is the fractional fluorescence due to the exposed residues.

In a process of dynamic quenching a plot of $I_0/(I_0 - I)$ vs. $1/[Q]$ gives a straight line with the intercept equal to $1/F_w$ (Eftink and Ghiron 1981; Lehrer 1971). When using this method, it is necessary to keep in mind the fact that F_w corresponds to the total fluorescence fraction resulting from the exposed Trp residues, which does not coincide with the fraction of Trp residues exposed. This is due to the fact that generally the fluorescence quantum yield of the buried residues is higher than that of the buried residues (Burstein et al. 1973; Eftink and Ghiron 1982).

In order to find a relation between the fluorescence fraction corresponding to the exposed Trp, F_w, and the fraction of the total Trp residues that are exposed, f_1, the following analysis was carried out:

$$F_w = \frac{I_w}{I} = \frac{n_w \phi_w}{n_w \phi_w + n_x \phi_x} \tag{3}$$

$$F_x = \frac{I_x}{I} = \frac{n_x \phi_x}{n_w \phi_w + n_x \phi_x} \tag{4}$$

$$\frac{F_w}{F_x} = \frac{n_w \phi_w}{n_x \phi_x} \tag{5}$$

where I is the total fluorescence intensity, n_w, I_w, ϕ_w, n_x, I_x, and ϕ_x are the number of residues, fluorescence intensities and fluorescence quantum

Table 1. Molecular weights (M.W.), maximum fluorescence emission and quenching parameters for tryptophan, and tryptophan containing proteins and peptides

	M.W.[a]	λ_{max} (nm)	K_{sv}[b]	F_w[c]
Trp	204	358	11.67	–
Trp-Urea	204	358	7.80	–
Trp-GuHCl	204	358	5.13	–
α-Lactalbumin	14,200	330	0.00	0.00
α-Lactalbumin-Urea	14,200	350	2.00	1.00
α-Lactalbumin GuHCl	14,200	355	2.53	1.00
α-Lactalbumin Red. and Carbox.	14,200	350	9.90	0.78
α-I	10,320	350	2.96	0.68
α-II	3,890	355	2.68	1.00
Lysozyme	14,600	342	2.78	0.52
Lysozyme-Urea	14,600	350	1.59	0.61
Lysozyme GuHCl	14,600	355	2.28	1.00
Lysozyme Red. and Carbox.	14,600	350	5.10	0.80
L-II	10,650	350	3.03	0.90
L-III	2,860	355	3.75	1.00

[a] Molecular weights (M.W.) were calculated from the aminoacid composition
[b] K_{sv} Stern-Volmer quenching constant
[c] F_w Fluorescence fraction corresponding to the exposed residues

yields corresponding to the exposed residues (w) and to the buried residues (x), respectively.

F_w and F_x are experimentally obtained from the Lehrer plot. If ϕ_w and ϕ_x are known, we have a relation between n_w and n_x, and with the number of total residues n_t, n_w and n_x can be calculated. f_1 can be determined:

$$f_1 = \frac{n_w}{n_w + n_x} = \frac{n_w}{n_t}. \tag{6}$$

Results and discussion

The wavelenght of maximum emission of fluorescence of Trp depends on the polarity of the medium (Burstein et al. 1973; Cowgill 1967; Sun and Song 1977). In aqueous medium the indolic ring of Trp emits with a maximum at 358 nm, and this wavelenght is not modified by the presence of 8 M urea or 6 M guanidine hydrochloride (Table 1). The fluorescence intensity of Trp in free form can be quenched by the presence of iodide. The efficiency of this quenching process is given by its K_{sv} value (shown in Table 1). The four Trp residues in α-lactalbumin are characterized by a fluorescence emission band at 330 nm, which would correspond to residues located in the interior of the protein structure.

When this protein is treated with denaturing agents, such as 8 M urea or 6 M guanidine hydrochloride, or is reduced and carboxymethylated, a conformational change is produced, which changes the fluorescence emission band to longer wavelengths. Figure 1 shows the effect of the addition

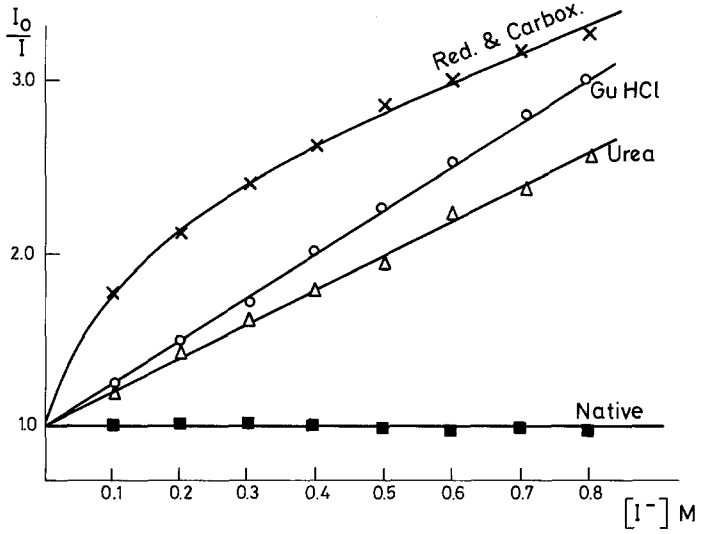

Fig. 1. Stern-Volmer plots of the fluorescence quenching of α-lactalbumin by iodide ion: native protein in 2 m*M* HEPES buffer (pH 7.5) (□); denatured protein in 6 *M* GuHCl 2 m*M* HEPES buffer (pH 7.5) (○); denatured protein in 8 *M* urea 2 m*M* HEPES buffer (pH 7.5) (△); reduced and carboxymethylated α-lactalbumin in 2 m*M* HEPES buffer (pH 7.5) (×)

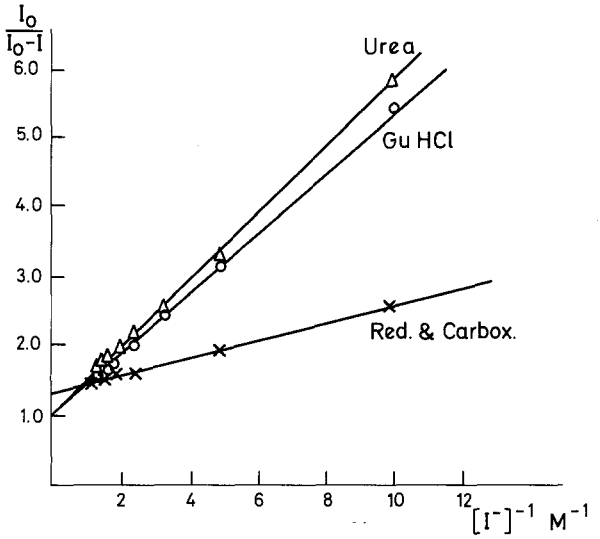

Fig. 2. Fluorescence quenching of modified α-lactalbumin by iodide ion, plotted according to the equation of Lehrer (4). Conditions as in Fig. 1

of iodide upon the fluorescence intensity of native and denatured forms of α-lactalbumin. From the Stern-Volmer plots shown in Fig. 1 it is inferred that the fluorescence of the Trp residues of native α-lactalbumin can not be quenched by iodide, which would confirm that these Trp residues are located in the interior of the protein. Figure 1 also shows that the fluores-

cence of the Trp residues in α-lactalbumin treated with 8 *M* urea or with 6 *M* guanidine hydrochloride can be completely quenched by the action of iodide as evidenced by the classical straight lines obtained in the Stern-Volmer representation. The fluorescence of the Trp residues of α-lactalbumin modified by means of reduction and carboxymethylation, can also be quenched in the presence of iodide; the fact that a straight line in the classical plot is not obtained, is typical of Trp residues with different degrees of accessibility to the quencher (Eftink and Ghiron 1981).

In order to determine the fraction of the total fluorescence that results from the Trp residues exposed to the solvent, the Lehrer equation was used. Plots are shown in Fig. 2. The intercept value corresponds to $1/F_w$, where F_w represents the fraction of the total fluorescence resulting from the exposed Trp residues. In the case of the reduced and carboxymethylated α-lactalbumin, 78% of the fluorescence is due to Trp residues exposed to the solvent.

By the action of CNBr and subsequent reduction and carboxymethylation, two polypeptide segments were obtained. Peptide α-1 (aa 1 to 90) contains two Trp residues (Trp-26 and Trp-60), which exhibit different degrees of exposure. This was recognized by the curvature obtained in the Stern-Volmer plot and by the value of $F_w = 0.68$ (Table 1) in the Lehrer equation. The other two Trp residues (Trp-104 and Trp-118) in α-lactalbumin which are present in the α-II segment are totally quenched, indicating that they are accessible to the solvent.

Table 1 also shows the values of λ_{max}, K_{sv} and F_w of native and modified lysozyme, and of peptides obtained by the action of CNBr on the enzyme (L-II aa 13-105 and L-III aa 106-129). The native lysozyme, the enzyme treated with 8 *M* urea, the reduced and carboxymethylated enzyme, as well as the peptide L-II, show in each case Trp residues with different degree of exposure, as previously demonstrated by means of photochemical methods (Edwards and Silva 1985; Ferrer and Silva 1981; Risi et al. 1973; Silva et al. 1974).

The fact that the fluorescence quantum yields of the Trp residues in a protein vary according to their location in the structure (Burstein et al. 1973; Churchich 1966; Eftink and Jameson 1982), complicates the direct calculation of the number of exposed residues from the corresponding value of F_w.

In order to estimate the number of buried and exposed Trp residues it was necessary to determine the fluorescence quantum yield for the residues of both types. The L-III segment (aa 106-129) of lysozyme shows three Trp residues (Trp 108, Trp-111 and Trp-129) which are exposed to the solvent according to its wavelength of maximum emission, $\lambda_{max} = 355$ nm (Table 1) and the value of $F_w = 1.0$ (Table 1) obtained from the Lehrer representation. These results are in good agreement with those obtained previously by photochemical methods (Ferrer and Silva 1981). It was possible to determine the fluorescence quantum yield of the L-III peptide whose Trp residues are considered representative of residues in contact with a polar medium. The determined value was $\phi_{Trp}^{Exp} = 0.05$ and was calculated

Table 2. Number of tryptophan residues, fraction of accessible fluorescence (F_w), fraction of exposed residues, (f_1), number of exposed (n_w) and buried (n_x) Trp residues in native and modified proteins and their peptides

	Trp	F_w	f_1	n_w	n_x
α-Lactalbumin	4	0	0	–	4.0
α-Lactalbumin Red and Carbox	4	0.78	0.59	2.4	1.6
α-Lactalbumin 8 *M* Urea	4	1.00	1.00	4.0	–
α-I	2	0.68	0.46	0.9	1.1
α-II	2	1.00	1.00	2.0	–
Lysozyme	6	0.52	0.30	1.8	4.2
Lysozyme Red and Carbox	6	0.80	0.62	3.7	2.3
Lysozyme 8 *M* Urea	6	0.61	0.38	2.3	3.7
L-II	3	0.90	0.78	2.3	0.7
L-III	3	1.00	1.00	3.0	–

from the known value of the fluorescence quantum yield for Trp in its free form, $\phi_{Trp}=0.14$ (Bent and Hayon 1975).

To calculate the fluorescence quantum yield corresponding to Trp residues that are buried in the central nucleus of the proteins, we made use of the Trp of β-lactoglobulin, whose $\lambda_{max}=333$ nm is typical of buried Trp residues. This is in agreement with the literature (Burstein et al. 1973). The fluorescence quantum yield value obtained was $\phi=0.02$ and the fluorescence intensity was not quenched by iodide ($F_w=0$). In the case of α-lactalbumin, which also shows $F_w=0$, a fluorescence quantum yield value of 0.024 was obtained.

The experimental values determined for the fluorescence quantum yields correspond well with those previously reported (Burstein et al. 1973; Churchich 1966) i.e., the quantum yields for the exposed Trp residues are higher than those corresponding to the buried residues. Once the quantum yields are known, it is possible to calculate the number of internal and external Trp residues starting from the corresponding values of F_w (See Materials and methods). The value ϕ_w/ϕ_x applied in the estimation of the data is an approximation, since Trp fluorescence yields (ϕ) do not only change when going from exposed (w) to buried (x) Trp. They also depend on the aminoacid composition around Trp (Cowgill 1963a, b, 1967). However, this value is in good agreement with those reported for the lifetime of the internal residues (τ_x) and those of the exposed ones (τ_w) in LADH, $\tau_w/\tau_x \sim 2.0$ (Ross et al. 1981; Eftink and Jameson 1982), if it is assumed that $\phi_w/\phi_x=\tau_w/\tau_x$. A good agreement is found as well with the values of τ reported by Churchich (1966) for lysozyme.

Table 2 shows the total number of Trp residues, the fraction of total fluorescence resulting from exposed residues (F_w), the fraction of Trp residues exposed to the solvent (f_1), the number of external (n_w) and internal (n_x) Trp residues, in native α-lactalbumin and lysozyme, in their conformationaly altered forms, and in fragments obtained by CNBr action. Analysis of the experimental values obtained in the case of lysozyme, a protein that

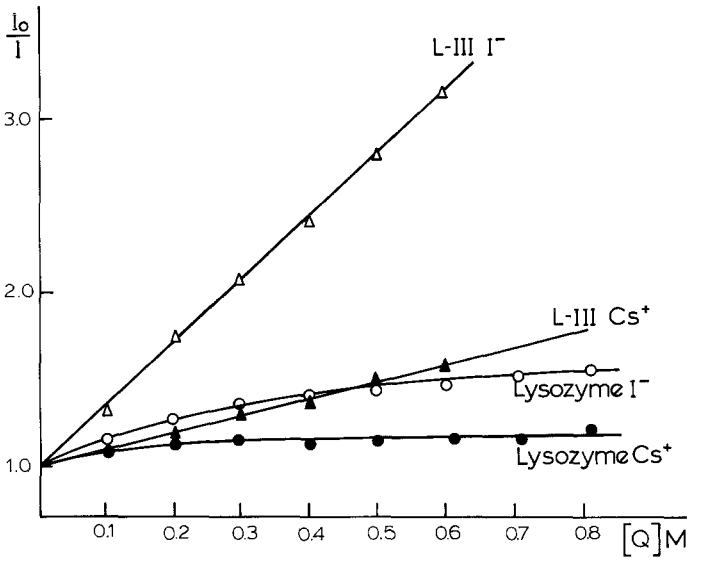

Fig. 3. Stern-Volmer plots of the fluorescence quenching of lysozyme and peptide L-III by iodide and cesium ion

can be considered as model since its tridimensional structure is known (Blake et al. 1967), shows that 52% of the total fluorescence of its six Trp residues comes from those residues that are in contact with the solvent. By the corresponding calculations it was determined that 30% ($f_1 = 0.3$; Table 2) of the total Trp residues present in lysozyme should be in the hydrophilic exterior. This means that of the six Trp residues, 1.8 correspond to external residues and 4.2 to internal ones. This result is in agreement with the fact that only Trp-62 and Trp-123 are exposed to the solvent (Blake et al. 1967; Edwards and Silva 1985; Hore and Kaptein 1983). The addition of 8 *M* urea or the reduction and carboxymethylation imply a conformational change that is expressed in an increase of the Trp residues exposed to the solvent. This confirms previous results obtained with photochemical methods (Edwards and Silva 1985).

Native α-lactalbumin lacks Trp residues the fluorescence of which could be quenched by ionic-type quenchers, but as its spacial conformation is altered, a greater number of Trp residues become exposed to the quencher.

Previous results obtained by means of sensitized photooxidation of native α-lactalbumin indicate that of the four Trp residues present in this protein, only one is exposed to the action of singlet oxygen (Edwards and Silva 1985). This result, different from the one obtained in this work when using iodide as quencher, can be explained by the fact that molecular oxygen is a small and uncharged molecule, which has been reported to be able to penetrate to the interior of proteins (Calhoun et al. 1983a, 1983b). When the α-lactalbumin structure is modified, an increase in the number of Trp residues accessible to singlet oxygen is observed in analogy to the behaviour shown with iodide.

The large peptide segments obtained by the action of CNBr upon lysozyme (L-II aa 13-105) and α-lactalbumin (α-I aa 1-90) show a conformation that allows the Trp residues to locate themselves in different environments.

In order to test whether the results obtained with an anionic quencher such as iodide could have been influenced by charge centers in the surface of the proteins, other series of experiments were carried out, employing cesium, a cationic quencher. The effects observed in this case were similar to the ones obtained with iodide, the only difference being the smaller efficiency of Cs^+ as quencher. As an example, Fig. 3 shows the effect of the Cs^+ concentration upon the fluorescence intensity of the Trp residues in lysozyme and in the L-III segment (aa 106-129), as compared with the effect produced by iodide. No effect was found on native α-lactalbumin fluorescence by either of the two compounds.

It can be concluded that an adequate interpretation of the values obtained when selectively quenching the fluorescence intensity of the Trp residues exposed to the solvent, allows a quantitative estimation of the number of these residues. This can give useful information about the structure of proteins in solution.

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