

BBA 35 006

FLUORESCENCE STUDIES OF THE MOLECULAR CONFORMATION
OF α -LACTALBUMIN

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(Received June 3rd, 1966)

SUMMARY

The ultraviolet fluorescence of α -lactalbumin was shown to be markedly altered during the 2 distinct conformational changes which this protein undergoes. The more subtle of these, which occurs at pH 6 as the temperature is lowered from 25° to 1°, involves contraction of the "crevices" housing the 2 'exposed' tryptophan groups and is accompanied by a short-wavelength shift of about 10 m μ of the emission spectrum. The more drastic process, denaturation below pH 4, results in a long-wavelength shift of 10-18 m μ and an increase in the apparent quantum yield of about 40%. The molecular origins of these spectral shifts have been interpreted in terms of a model derived from a recent theory which explains shifts in the emission spectrum of tryptophan in terms of an excited state complex. According to this model, the thermal transition which occurs at pH 6 for α -lactalbumin produces a short-wavelength shift due to inhibition of complex formation resulting from hindered rotation of tryptophans in the contracted "crevice". The low pH denaturation of α -lactalbumin, which we have shown earlier to involve molecular swelling but no enhanced exposure of tryptophans, produces a long-wavelength shift of the emission spectrum due to enhanced complex formation resulting from increased freedom of rotation of the 3 "buried" tryptophans.

INTRODUCTION

The fluorescence of the aromatic amino acids in proteins offers a particularly sensitive probe in detecting molecular conformational changes¹⁻⁶. Use of this technique, however, to define such change in terms of specific molecular processes has been comparatively limited due primarily to the fact that changes in the fluorescence characteristics of proteins potentially have such a wide variety of origins. This is particularly true of tryptophan fluorescence where solvent effects are known to have such a marked influence on the emission spectrum as compared to the absorption spectrum.

For some time now, we have been studying the milk protein, α -lactalbumin, and the several conformational changes that it undergoes⁷⁻¹⁵. There appear to be two rather distinct molecular changes for this protein, both of which involve alteration of the environment of tryptophan residues but in strikingly different ways:

(a) below pH 4 molecular swelling occurs^{8,15}; a tryptophan difference spectrum is generated^{9,14}; changes in optical rotation dispersion properties are observed^{11,13} and the protein becomes increasingly prone to aggregation and dissociation^{7,8}. Although a denaturation blue shift of the absorption spectrum is observed, solvent perturbation measurements indicate that no change in exposure of tryptophan groups occurs during the acid conformational change^{9,10}, *i.e.* three groups are completely buried and two are completely exposed. Comparable changes in physicochemical properties of α -lactalbumin suggest that an identical process occurs above pH 10 (refs. 13-15). (b) A more subtle conformational alteration is observed if the temperature of α -lactalbumin solution is lowered from 25 to 0-2° at pH 6 (see refs. 9, 10). Solvent perturbation measurements indicate that at the lower temperature the two exposed groups are no longer accessible to large perturbants such as sucrose molecules but remain fully available for small perturbants such as heavy water¹⁰. This process, which we have called "crevice contraction" is not accompanied by any change in absorption spectrum⁹.

In the present paper, we shall present some observations of the changes in the wavelength distribution and yield of tryptophan fluorescence from α -lactalbumin occurring during these two unique conformational alterations. Interpretation of these changes with the aid of collateral physicochemical information provides some idea of the kind of molecular processes which may lead to alteration of fluorescence properties of tryptophan-containing proteins.

EXPERIMENTAL

Materials and methods

Preparation No. R49 of α -lactalbumin was employed. *N*-acetyltryptophan ethyl ester and tryptophan were Mann products. In a few instances Schwartz optical-grade tryptophan was employed. Protein solutions were prepared from slurries of α -lactalbumin as previously described^{7,8}. A series of dilute solutions for fluorescence measurements at various pH values were prepared from the same stock solution. Concentrations of all fluorescent substances were chosen such that the absorbance of the final dilutions were less than 0.1. For α -lactalbumin, this corresponds to concentrations of the order of 5 mg per 100 ml. Methods for pH measurement and determination of α -lactalbumin concentrations have been described previously^{7,8}. A Cary Model-14 recording spectrophotometer was used for absorption measurements.

Fluorescence measurements

The fluorescence spectrometer employed has been previously described by WINKLER¹⁶. It makes use of a 450-W xenon lamp, a 1P28 photomultiplier, a Bausch and Lomb 500-mm grating monochromator for excitation and a prism monochromator derived from a Beckman DU spectrophotometer to disperse the emitted light. The monochromators were potentiometrically coupled to the x axis of an x-y recorder to permit scanning of excitation and emission spectra.

Measurements were made in 1-cm path length cells fabricated of low fluorescence quartz (Pyrocell Corp.). Cells were thermostated in a two place sample compartment maintained to $\pm 0.5^\circ$. In order to minimize the effect of instrument drift, fluorescences of samples were compared at frequent intervals with that from a solution

of *N*-acetyltryptophan ethyl ester of comparable intensity. In experiments where yields at various temperatures were obtained relative to those at 25°, the standard tryptophan ester solution was maintained in an external cell holder maintained at 25 ± 0.5° and quickly transferred to the cell compartment for comparison with the protein solution at the temperature in question.

Apparent relative quantum yields of α -lactalbumin at various pH values were calculated using a pH-5.8 to pH-6 solution as unity. These were calculated for each set of dilutions prepared from a single stock solution (see above) using the equation given by PARKER AND REES¹⁶ for dilute solutions:

$$Q = \frac{A_{\text{pH}}(D)_{\text{pH } 6}}{A_{\text{pH } 6}(D)_{\text{pH}}}$$

where A_{pH} is the area under the emission curve at the pH in question, $A_{\text{pH } 6}$ is the area of the pH-6 emission curve and $D_{\text{pH } 6}$ and D_{pH} are the respective absorbances determined in the fluorescence cell. Since the protein concentrations within a dilution series are identical, the absorbance differences between an acid and a pH-6 solution were of the order of 10–15% (ref. 9). Thus the uncertainty inherent in the use of the above approximate equation rather than the exact equation¹⁶ is less than 5%. This is comparable to the experimental error.

RESULTS

Emission spectra of α -lactalbumin at 25°

Fluorescence spectra obtained for α -lactalbumin are typical of those generally observed for tryptophan-containing proteins, *i.e.* the spectra resemble that of tryptophan. Although α -lactalbumin contains five tyrosine and five tryptophan residues per molecule¹⁷, there seems to be little if any contribution from the former amino acid. As has been observed by TEALE² and others for tryptophan-containing proteins, the emission spectra are shifted to lower wavelengths as compared to the free amino acid. In the case of native α -lactalbumin (pH 6), the emission maximum occurs at about 342 m μ , as compared with 360 m μ to 363 m μ found for tryptophan and *N*-acetyltryptophan ethyl ester (Table I)*.

Emission spectra of α -lactalbumin on acid denaturation are shifted toward the red as compared to pH 6 and the yields are increased markedly. These effects occur quite rapidly, such that by the time the solution is transferred to the instrument (about 1 min) the changes in fluorescence are already complete. In no case were there further changes up to periods of 2 h. The pH dependence of the apparent yield and of the emission maximum at 25° are shown in Fig. 1 and Curve a of Fig. 2.

* The fact that the emission maximum of tryptophan and *N*-acetyltryptophan ethyl ester are higher than the literature values of 348–355 m μ (see refs. 1, 19, 20) seems to be due to the fact that no correction was made for monochromator efficiency or photomultiplier response. A comparable difference was also observed by us for emission maximum for bovine serum albumin, ovalbumin, and indole in aqueous media. After this study was completed, we were able to determine emission spectra of α -lactalbumin using the Turner absolute fluorescence spectrometer and found λ_{max} to be 10–12 m μ lower than those reported here (see Table I). Nonetheless, the position of the emission maxima should probably be taken to have only semi-quantitative significance as should the relative quantum yields.

Over the pH range 6 to 3.5 the yield is essentially constant, whereupon an increase of about 40% occurs over less than one pH unit (Fig. 1). The pH dependence of the emission maximum is more complex (Curve a, Fig. 2). λ_{max} increases with decreasing pH, goes through a minimum at the isoelectric point (about pH 4.75)¹⁵ and then increases above pH 4.5 to attain a final value of 350–352 m μ above pH 3.

TABLE I

EMISSION MAXIMA FOR α -LACTALBUMIN AND TRYPTOPHAN

All solutions in 0.15 M KCl, temperature 25°.

Substance	λ_{max} (m μ)
Native α -lactalbumin (pH 6)	342
Acid-denatured α -lactalbumin (pH 2)	350
Urea-denatured α -lactalbumin (pH 2.7 to 6.5)	353
Tryptophan (neutral pH)	360*, 362**
N-acetyltryptophan ethyl ester	363

* Mann preparation.

** Schwartz preparation.

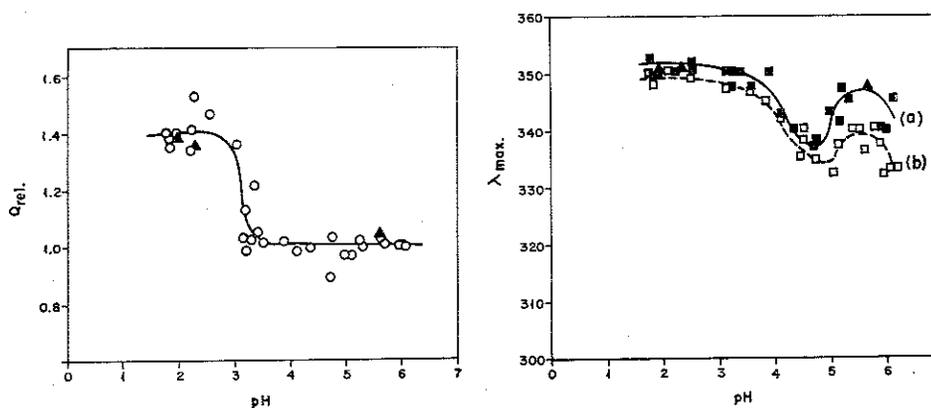


Fig. 1. pH dependence of apparent quantum yield (Q_{rel}) of α -lactalbumin at 25°. Yields are taken relative to value at pH 6. \circ , 0.15 M KCl; \blacktriangle , salt-free solutions. Protein concn. about 0.005 g per 100 ml.

Fig. 2. pH dependence of the emission maximum, λ_{max} for tryptophan fluorescence of α -lactalbumin. \square , 0.15 M KCl; \blacktriangle , salt-free. Other conditions as in Fig. 1. Curve a, 25°; Curve b, 4°.

The changes in fluorescence illustrated in Figs. 1 and 2 are completely reversible. Solutions of α -lactalbumin exposed to pH 2 for 1 to 2 h and readjusted to pH 6 gave yields and emission spectra identical with the original pH-6 solution. No attempt was made to study the kinetics or a possible pH dependence of the reversal process.

These alterations of the fluorescence properties of α -lactalbumin correspond only roughly to those observed in the tryptophan absorption spectrum although an exact comparison is impossible since the latter measurements could not be extended above pH 4 at 25° (see ref. 9). About 75% of the change in the difference extinction coefficient, $\Delta\epsilon_{293\text{ m}\mu}$, occurs between pH 4 and 3.5, in contrast with the displacement

to higher pH values of the transition as seen by increases in yield (Fig. 1). The increase in λ_{\max} , on the other hand, begins above pH 4 (Curve a, Fig. 2). Both yield and λ_{\max} are independent of ionic strength as seen from the data obtained with salt-free solution adjusted to the appropriate pH with 1 M HCl (filled triangles, Fig. 1; Curve a, Fig. 2). The insensitivity of the conformational change to ionic strength has been previously noted by measurement of difference spectra⁹ and by optical rotation dispersion¹³.

Emission spectra of α -lactalbumin at 4°

Changes in λ_{\max} with decreasing pH at 4° parallel those observed at 25° with the low temperature curve being displaced, however, toward somewhat shorter wavelengths, particularly above pH 5 (compare Curves a and b, Fig. 2). This displacement with decreasing temperature is clearly seen on comparison of the spectra themselves (Fig. 3). These were obtained on successive scans of a single protein solution at the two temperatures. In all cases the original spectrum could be regained by returning the solution to its original temperature.

At pH 6.08 (Fig. 3a), λ_{\max} shifted from about 345 m μ to 332 m μ as the temperature was lowered, while smaller blue shifts were observed at pH 4.72 (Fig. 3b) and pH 3.65 (Fig. 3c). The shift at pH 1.83 (Fig. 3d) although small, is probably real in view of the general downward displacement of the 4° curve below pH 3.25 (compare

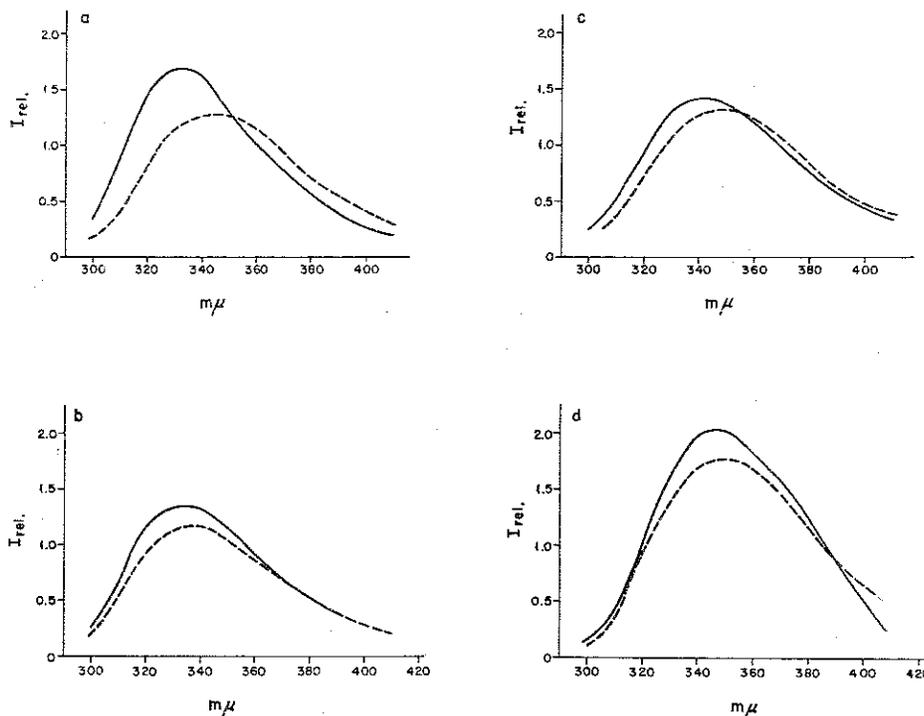


Fig. 3. Tryptophan emission spectra of α -lactalbumin as a function of temperature and pH. Conditions as in Fig. 1. The intensities are in arbitrary units but have been normalized such that the areas at a given pH are proportional to the relative yields at the two temperatures (—, 4°; ----, 25°). a, pH 6.08; b, pH 4.72; c, pH 3.65; d, pH 1.83.

Curves a and b, Fig. 2). Shifts in the emission spectra with decreasing temperature were not observed for tryptophan or *N*-acetyltryptophan ethyl ester in agreement with the observations of GALLY AND EDELMAN⁶.

pH dependence of apparent quantum yield at 4°

The yield *versus* pH curve at 4° differs significantly from that observed at 25° (compare Figs. 1 and 4). Between pH 5 and 6 the yield (relative to pH 6, 4° as unity) decreased by about 15%, a minimum lying between pH 4 and 5. This minimum appears to be associated with that observed in the λ_{\max} *versus* pH curve (Curve b, Fig. 2). These minima are not due to association or aggregation of α -lactalbumin in the isoelectric region, a possibility which suggested itself on the basis of the known solubility and aggregation behavior of this protein^{7,8}. Measurement of fluorescence as a function of protein concentration over the range 0.002 to 0.01 g per 100 ml yielded a constant intensity/concentration ratio. The minima in the curves of Figs. 2 and 4, therefore, must be a reflection of changes in the molecular environment of tryptophan groups in the monomeric molecule.

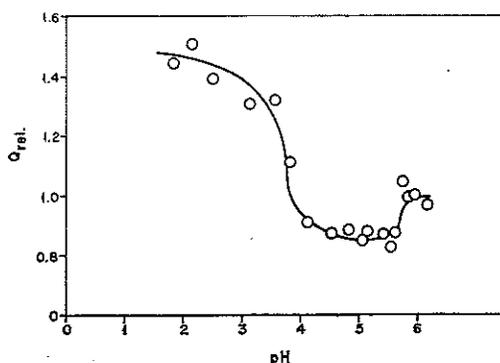


Fig. 4. pH dependence of apparent quantum yield of α -lactalbumin at 4°. Yields are taken relative to that at pH 6. Conditions as in Fig. 1.

The increase in yield at 4° begins below pH 4 and is more than 60% complete by pH 3.5, whereas at 25° the relative yield was still unity at the latter pH. The pH dependence of the yield at 4° more nearly resembles that of $\Delta\epsilon_{293\text{ m}\mu}$ (ref. 9) than did the 25° data. The increase in yield on acid denaturation (pH 6 \rightarrow pH 2) was about 40–45%, comparable to that observed at 25° (compare Figs. 1 and 4). In view of the quenching occurring in the isoelectric region at 4°, however, this comparison may not be valid.

Effect of temperature on intensity of fluorescence

Although the temperature dependencies of λ_{\max} (Figs. 2 and 3) are evidence of conformational changes in α -lactalbumin, plots of fluorescent intensity *vs.* temperature at pH 6 and below were linear within experimental error in the range 0 to 25° and showed no breaks comparable to those observed by STEINER AND EDELHOCH^{3,4} and GALLY AND EDELMAN⁶ on thermal denaturation of a variety of proteins. These observations do not preclude the possibility, however, that the changes in fluorescence

associated with thermally induced conformational changes occur over a sufficiently wide range to be obscured by the normal thermal quenching.

Excitation spectra

Excitation spectra for α -lactalbumin solutions did not reveal the conformational changes at acid pH. Although emission spectra showed shifts of 10 to 18 m μ in going from pH 6 to 2 (see Figs. 2 and 3), neither the position nor half band width of the excitation spectrum showed a comparable change, the maximum occurring at 286 ± 1 m μ . This was about 6 m μ higher than the value observed for free tryptophan in neutral solution. The insensitivity of excitation spectra to environmental changes has been also noted by VAN DUUREN²¹ in his study of the effect of solvents on indole fluorescence.

Urea denaturation of α -lactalbumin

Treatment of α -lactalbumin with 8 M urea at 25° resulted in large increases in yield and a red shift of the emission maximum (Table I) relative to the native protein. As has been previously observed for difference spectra⁹ and for optical rotation dispersion^{11,13}, these fluorescence changes in urea were instantaneous and showed no pH dependence, in contrast with the behavior of urea-free protein (Fig. 1; Curve a, Fig. 2). The apparent relative yield was about 2.4, considerably larger than the value of 1.4 observed on acid denaturation (Fig. 1). The magnitude of the former increase was comparable to those reported by TEALE² for a variety of proteins. The difference in λ_{\max} between acid and urea-denatured α -lactalbumin, while only 3 m μ , is experimentally significant, the entire spectrum being displaced toward higher wavelengths in the latter case. The emission maximum, 353 m μ , obtained for urea-denatured protein is significantly lower than the value of 360 m μ to 363 m μ found for tryptophan (Table I), in contrast with TEALE'S² observation that urea-denaturation of proteins generally shifted emission maxima close to that of the free amino acid.

DISCUSSION

Tryptophan fluorescence in proteins

While it is apparent that a variety of molecular parameters can influence the yield and spectral distribution of tryptophan fluorescence in proteins, considerable simplification seems possible by interpretation in terms of a model derived from recent fluorescence studies of tryptophan, indole and indole derivatives* and from solvent perturbation studies of proteins^{10,22-25}. The protein model inferred from the latter type of studies is that chromophores such as tyrosine and tryptophan can be divided into two classes: "buried" and "exposed". Such a distinction is an operational one and will depend upon the choice of perturbant, *e.g.* it may not be possible to distinguish the partial burying of a large number of groups from complete burying of some and complete exposure of others. This ambiguity can, however, often be removed by comparing the results obtained with a variety of perturbants having different sizes and "range effects" (see for example, ref. 10). To a first approximation we may assume a difference in polarity of the immediate surroundings of buried and exposed groups.

* R. LUMRY, S. YANARI AND F. A. BOVEY, unpublished manuscript of paper presented at the International Congress of Photobiology, Oxford, England, August 1964.

The importance of distinguishing between buried and exposed groups is evident from studies of VAN DUUREN²¹ and of LUMRY, YANARI AND BOVEY* who have observed that the wavelength distribution of fluorescence of indole and indole derivatives is markedly dependent on the polarity of the medium. LUMRY, YANARI AND BOVEY* found, however, that when ethanol was added to a solution of *N*-acetyltryptophan ethyl ester in dry cyclohexane, the fluorescence maximum shifted from 303 m μ reaching a value of 335.8 m μ in the range 2–8 % ethanol. About 80 % of this shift occurred after addition to concentrations of only 2 %. Above a concentration of 8 %, a small decrease in emission maximum was noted such that in pure ethanol the emission maximum had a value of 334.3 m μ . The latter shift was interpreted as being due to a non-specific solvent effect (see ref. 3 and subsequent discussion). They further conclude that the larger red shift is due to a new fluorescent species—a complex of a polar molecule and the excited indole nucleus. Uncomplexed indole (non-polar medium) has an emission maximum of about 297 m μ while that for water complexed indole lies at about 350 m μ . Shifts in emission maxima observed on various treatments of tryptophan-containing proteins, according to this mechanism, would be due to changes in complexing of these chromophores*.

It is difficult to reconcile the above observations with any theory of non-specific solvent effects such as the dipole relaxation mechanism of spectral shifts described by LIPPERT²⁶ and applied by MATAGA²⁷ to tryptophan and indole. This theory predicts that the magnitude of the spectral shift is dependent upon the dielectric constant and refractive index of the medium. Clearly, the concentration dependence of the spectral shifts cited above cannot be the result of alterations of refractive index or dielectric constant of the medium.

With the availability of information on the distribution of tryptophan groups in α -lactalbumin¹⁰ a tentative model can be formulated which will account for many of the fluorescence properties of this protein. This model may not necessarily be unique, however, in interpreting these observations. To provide a framework for such a model, a brief digression concerning fluorescence properties of indole, tryptophan and tryptophan in protein seems in order. The wavelength distribution of fluorescence from a tryptophan-containing protein will, according to the complexing hypothesis described above, depend upon: (a) The relative number of exposed and buried tryptophan groups. The exposed residues should readily complex with molecules since the life time of the excited state (10^{-8} to 10^{-9} sec) is large as compared to the time for rotation or vibration (about 10^{-12})²⁸ that might be required to yield the requisite geometry for complex formation. Such tryptophans should have the emission characteristics of tryptophan in water (λ_{\max} about 350 m μ). Buried tryptophans in non-polar regions of the molecule would be expected to have emission characteristics comparable to that of indole in non-polar solvents (λ_{\max} about 300 m μ). The emission characteristics of buried tryptophans in non-polar environments but in close proximity to relatively polar groups, such as the peptide linkage, will depend upon the freedom of rotation of such residues. If vibration or rotation is capable of producing the geometry requisite for complex formation during the lifetime of the excited state, the emission characteristic will be that of complexed indole ($\lambda_{\max} > 300$ m μ). (b) The relative magnitudes of quantum yields of buried and exposed groups.

* R. LUMRY, S. YANARI AND F. A. BOVEY, unpublished manuscript of paper presented at the International Congress of Photobiology, Oxford, England, August 1964.

Little information is available as to the efficiency of quenching of tryptophan incorporated into a protein. It is therefore difficult to compare the relative intensities of fluorescence anticipated for such groups. (c) The efficiency of transfer of energy among the different tryptophan groups. The probability of such transfer is high since R_C (the characteristic distance between chromophores in which the probability of transfer and emission are equal) is of the same order of magnitude as the dimensions of the protein molecule²⁹. Since the absorption band of complexed tryptophans overlaps the emission band of uncomplexed tryptophans ($\lambda_{max} < 300 m\mu$), transfer from uncomplexed to complexed residues would be anticipated²⁸. Similarly, since the absorption band of uncomplexed tryptophans and the emission band for complexed tryptophans shows much less overlap, the probability of transfer of the excited state from complexed to uncomplexed tryptophans is low[§]. The net effect of transfer of the excited state should be that the emission spectrum of the protein will reflect complexed tryptophans to a greater degree than uncomplexed groups.

Molecular changes in α -lactalbumin at pH 6

In terms of the model outlined above, the shift of the emission maximum of α -lactalbumin from 340–342 $m\mu$ to 332 $m\mu$ as the temperature is decreased from 25° to 4° (Fig. 3a; Table I) would be the reflection of a structural change which decreases the extent of tryptophan complex formation. The molecular alteration at this pH must be rather limited in scope since it is not accompanied by a change in the ultraviolet absorption spectrum⁹ or in the rotatory dispersion parameters¹³. Likewise, ultracentrifuge measurements at pH 6 (see ref. 7) did not detect any changes in hydrodynamic properties in going from 25° to 10°. Solvent perturbation measurements, on the other hand, do show a difference in the exposure of tryptophan groups¹⁰. Those observations are summarized in Table II.

TABLE II
EXPOSURE OF TRYPTOPHAN GROUPS IN α -LACTALBUMIN

Protein	Tryptophans* exposed	
	Large perturbants	² H ₂ O
1. Native (pH 6, 25°)	2**	2
2. Acid-denatured (pH 2–3, 25°)	2**	2
3. Native (pH 6, 1°)	0***	2
4. Acid-denatured (pH 2–3, 1°)	1***	2
5. Urea-denatured (pH 2.6–6, 25°)	4**	—

* Taken from ref. 10, all solutions in 0.15 M KCl. Exposure based on a total of five tryptophan groups.

** Perturbants: 20% sucrose, glycerol, ethylene glycol.

*** Perturbants: 20% sucrose, glycerol.

All perturbants employed in the latter study (sucrose, glycerol, ethylene glycol and heavy water) revealed that of the five tryptophans in α -lactalbumin, three are

§ Fluorescence polarization spectra measured by WEBER^{29,30} are consistent with the hypothesis of the transfer of the excited state amongst tryptophans in proteins. These measurements showed that transfer occurred in solutions of the free amino acid²⁹. Similar measurements made with tryptophan-containing proteins³⁰ showed significant depolarization of the fluorescence at wavelengths at which transfer depolarization was observed with the free amino acid.

buried and two are exposed at 25° near pH 6. As the temperature is lowered to 1°, the latter two groups become completely buried with respect to sucrose and glycerol but remain exposed to heavy water. We suggested that the two groups exposed at 25° lie in crevices having as a critical dimension a distance greater than 9.4 Å (the effective diameter of the largest perturbant, sucrose) and that at low temperature the crevice contracts¹⁰. The size of this contracted crevice would be small enough to exclude molecules as large as sucrose and glycerol (effective diameter, 5.4 Å) but large enough to admit water molecules (effective diameter, 2 Å). This model is illustrated in Figs. 5a-c for a single tryptophan group. In accordance with the observations of YANARI AND BOVEY³¹, we have depicted the water molecule as hydrogen-bonded to the indole nitrogen (Fig. 5a). In order for the excited state-complex to form, the latter hydrogen bond must be broken, a process favored by the increased electro-

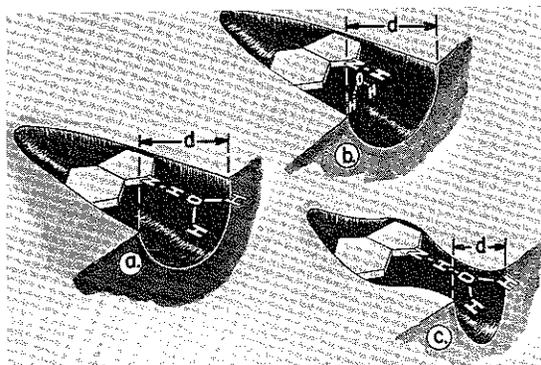


Fig. 5. Highly schematic view of a crevice housing an exposed tryptophan group. The crevice has been cut away to permit the group to be seen. The distance, d , represents a critical dimension estimated from solvent perturbation measurements¹⁰. a. pH 6, 25°, ground state. Water molecule hydrogen-bonded to indole hydrogen. $d > 9.4$ Å. b. pH 6, 25°, excited state. The water molecule has moved into close proximity with the indole nitrogen to form the excited state complex. The dashed bonds lie below the plane of the indole ring. The dimensions of d are the same as in Fig. 5a. c. pH 6, 4°, ground and excited states. The crevice has contracted, thereby preventing reorientation of the water molecule to form the excited state complex. $5.4 \text{ Å} > d > 2 \text{ Å}$.

positive character of the nitrogen in the excited state, and the water molecule must move to the appropriate complexing site on the indole nucleus. In Fig. 5b we have depicted this as the indole nitrogen, although the position on the ring is not critical for our argument. In this case complexation would occur through interaction of the electronegative water oxygen atom and the electropositive nitrogen of the excited indole nucleus.

At 25° the size of the crevice would permit rotation of the tryptophan side-chain or reorientation of the water molecule during the lifetime of the excited state so as to provide the requisite geometry for complex formation (Fig. 5b). At 4°, on the other hand, (Fig. 5c), rotation and reorientation would be impeded in the contracted crevice making complex formation less probable. Such a decrease in complexing at 4° according to the model outlined above, would account for the blue shift of the emission spectrum (Figs. 2 and 3a). The absence of a shift of the absorption spectrum is consistent with the above mechanism. The position of an absorption band depends, at least in part, on the polarizability of the medium surrounding

a chromophore in its ground state (see for example, YANARI AND BOVEY³¹). Since the crevices housing the two exposed tryptophans are penetrable to water molecules both before and after contraction, the polarizability should remain essentially constant during this structural change.

Molecular changes in α -lactalbumin at low pH

The acid denaturation of α -lactalbumin occurring below pH 4 involves a more drastic conformational change than the thermally induced transition at pH 6. An increase in the frictional ratio from about 1.0 (neutral pH region) to 1.3 (pH 2) indicates a more swollen or elongated molecule⁸. Titration curve data¹⁵ indicates that this swelling occurs in the spectral transition region (pH 3-4)⁹. Although the absorption spectrum of the denatured protein (below pH 3) is blue shifted relative to the native state (pH 6) at 25°, no additional groups become exposed (compare Lines 1 and 2, Table II)^{9,10}. For reasons discussed earlier^{9,10} it seems likely that the absorption changes are the result of environmental changes involving the three buried groups.

These environmental changes appear to include increased freedom of rotation of the buried tryptophans. In the native state side-chain Cotton effects occur in the tryptophan absorption region which are abolished on acid denaturation^{11,13}. The dependence of the change in optical rotation dispersion properties is comparable to that observed for the tryptophan difference spectrum⁹ and for the fluorescence properties (see Figs. 1 and 2). The abolition of the tryptophan Cotton effect is consistent with increased freedom of rotation of residues which had formerly been "frozen" into specific configurations (see refs. 11 and 13 for more complete discussion of this point).

Thus, polar groups and buried tryptophans should be freer to rotate than in the native state, such that the probability of complex formation between such groups is enhanced. The long-wavelength shift at 25° of the emission maximum below pH 4 (Curve a, Fig. 2) is consistent with this hypothesis. The shift of the emission maximum with decreasing pH at 4° (Fig. 2b) probably also involves the three buried groups. This uncertainty is due to the exposure of a single group in going from pH 6 to pH 3 and below (compare Lines 3 and 4, Table IV). Unfortunately, for reasons discussed earlier¹⁰, significant solvent perturbation measurements cannot be made in the transition region (pH 3 to 4) and we cannot determine if this exposure occurs above or below pH 4. The higher degree of exposure of tryptophans in the acid-denatured protein at 25° (compare Lines 2 and 4, Table II) is consistent with the long-wavelength displacement of the λ_{\max} versus pH curve below pH 3 (Curves a and b, Fig. 2).

pH dependence of the fluorescence changes

As we have considered above, α -lactalbumin is capable of undergoing two distinct conformational changes, crevice contraction and the low-pH denaturation. The overall pH dependencies of the yield (Figs. 1 and 4) and the emission maximum (Fig. 3), might therefore, represent the superposition of these two processes. Since solvent perturbation measurements cannot be made much above pH 3 or below pH 6 (ref. 10), the degree of overlap of these two molecular events can only be surmised. In any event, the complex character of the λ_{\max} versus pH curve (Fig. 2) and the quenching of the fluorescence at low temperature in the isoelectric region (Fig. 4)

indicate that these molecular alterations begin at much higher pH than would have been anticipated on the basis of the changes in absorption spectra⁹ or of rotatory dispersion parameters^{11,13}.

While direct verification is impossible, it seems reasonable to identify the minima in the λ_{\max} versus pH curves with contraction of the tryptophan-containing crevices. The fact that the pH dependencies of the yield are so different at 4° and 25° (compare Figs. 1 and 4) makes it unlikely that the fluorescent changes in the isoelectric region are of non-specific origin such as ionization of carboxyl groups, since the latter process occurs with almost zero enthalpy¹⁵. The ability of such crevices to contract as the temperature is lowered (see Table II and previous discussion) at pH 6 (protonic charge = -6) makes it likely that even more restricted rotation of water molecules and side chains will occur within these crevices at the isoelectric point (pH 4.75)¹⁵. The decreased value of λ_{\max} (Curve b, Fig. 2), therefore, could be a reflection of a further decrease in probability of complex formation. Similarly, more intimate contact between tryptophans and other side-chains within the crevice might be expected to produce quenching in the isoelectric region relative to pH 6 (Fig. 4), but its absence in the yield curve (Fig. 1) would indicate a more limited isoelectric crevice contraction than at 4°. We raised the question earlier⁸ as to whether the low isoelectric solubility of α -lactalbumin might be due to a particularly favorable distribution of polar and non-polar side-chains at the surface of the native protein molecule or whether this distribution might be the consequence of a subtle conformational change occurring in this pH region. While the former alternative cannot be excluded, the pH dependence of λ_{\max} and of the yield at low temperature clearly indicates a structural alteration in the isoelectric region.

The pH dependencies of λ_{\max} at 4° and 25° (Fig. 2) appear to follow the same course, although the former curve is shifted to shorter wavelengths. By contrast, the yields at 4° and 25° have significantly different pH dependencies (Figs. 1 and 4), the increase in apparent yield at the higher temperature occurring more than 0.5 pH units lower. We have pointed out earlier⁹ that the transition in the pH region 3 to 4 probably involves simultaneous or closely consecutive alterations of the molecule in regions adjacent to different buried tryptophan groups. The lack of correspondence of the 4° and 25° yield curves may thus be a reflection of this complex character of the transition.

Urea denaturation of α -lactalbumin

The shift of λ_{\max} for urea-denatured α -lactalbumin relative to native protein (Table I) is in the direction anticipated if the three buried tryptophans become "exposed" and are now free to complex with water molecules. Similar shifts have been observed by TEALE² on urea denaturation of a variety of proteins, but in contrast with our observations, λ_{\max} was generally comparable with the value found for free tryptophan groups. This difference that we observed with α -lactalbumin may be due to incomplete disruption of the molecule such that tryptophan residues continue to be insulated from the medium. The observation of a near-zero value for the rotatory dispersion parameter, b_0 , indicates however, that the molecule is not actually refractory toward urea denaturation¹³. The 80% exposure seen by solvent perturbation could correspond to either: (a) four groups completely exposed (Line 5, Table II), or (b) two groups completely exposed plus three groups exposed to the extent of

about 70%. The low value of λ_{\max} for urea-denatured α -lactalbumin suggests that the first of these is probably true.

Spectral changes and protein conformation

The observations reported in this study are a compelling illustration of the value of complete emission spectra in detecting structural changes in proteins, as against the exclusive use of intensity measurements at a particular wavelength. This is likely to be of particular importance in determining the effect of temperature on a protein where normal non-specific thermal quenching is likely to obscure any change in yield. This was apparently the case for the pH-6 thermal transition of α -lactalbumin where a shift of nearly 10 m μ occurred in the emission spectrum without any break in a yield *versus* temperature curve being obvious.

It is quite apparent from our findings that comparatively large alterations in fluorescence properties can occur without these necessarily being the result of drastic changes in the folding of the molecule. Indeed, a process as limited in scope as the pH-6 crevice-contraction observed for α -lactalbumin can lead to significant changes. It would appear that with our present state of knowledge, interpretation of changes in emission properties of a protein in terms of any specific mechanisms is quite hazardous in the absence of ancillary information of the type provided by solvent perturbation. A similar conclusion has been reached concerning the interpretation of changes in absorption spectra⁹. The observations made in this study, together with those reported in previous papers^{9,10}, illustrate the value of the simultaneous application of absorption, emission and solvent perturbation spectral techniques in characterizing conformational changes in proteins. It would appear that the tryptophan-complexing mechanism proposed by LUMRY, YANARI AND BOVEY* may provide a useful frame of reference in this regard.

ACKNOWLEDGEMENTS

I wish to thank Dr. R. B. PENNELL for extending me the hospitality of the facilities of the Protein Foundation where these measurements were carried out. A particular debt of gratitude is owed to Dr. M. WINKLER for making his fluorescence spectrometer available, as well as his continued assistance throughout the course of the experimentation. Many of the ideas regarding interpretation of the fluorescence data for α -lactalbumin came about through discussions with Dr. R. LUMRY and Dr. R. BILTONEN, as well as through an opportunity to examine a preliminary draft of a manuscript on tryptophan fluorescence in proteins by Drs. R. LUMRY, S. YANARI AND F. BOVEY. I am especially indebted to Dr. LUMRY for his assistance in this regard.

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