MOLECULAR CONFORMATION AND FLUORESCENCE PROPERTIES OF α LACTALBUMIN FROM FOUR ANIMAL SPECIES

P.B. Sommers* AND M.J. Kronman
Biochemistry Department, Upstate Medical Center, State University of New York, Syracuse, New York

AND

K. Brew
Department of Biochemistry, University of Leeds, Leeds 2, England

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Summary

The quantum yield of tryptophan fluorescence of guinea-pig α lactalbumin is more than twice as large as found for the bovine, goat, or human proteins. This difference is due to the absence of Trp 60 from the guinea-pig amino acid sequence. It is proposed that the presence of this residue results in marked quenching of a second tryptophan residue for the other three proteins. Reference to the "lysozyme analogy model" suggests that the quenched residue is most likely Trp 104, which lies within 7Å of Trp 60, the latter lying within 6Å of two disulfide bridges. It is suggested that transfer of the excited state energy takes place from Trp 104 to 60 with subsequent quenching by the two vicinal disulfide bridges. These observations provide support for the validity of the "lysozyme analogy model."

Introduction

The observation that bovine α lactalbumin and hen's egg white lysozyme possess a high degree of sequence homology (1-4) led Brew and coworkers to conclude that the three-dimensional folding of the two protein molecules must be very similar. Model building experiments subsequently showed that a reasonable molecular model of α lactalbumin could be built by folding the α-lactalbumin amino acid sequence to the known conformation of the lysozyme backbone (5). Since then a number of studies (6-15) have been published whose aim has been to test the validity of the "lysozyme analogy model"; most of these observations are consistent with the predictions of the model. Many of these studies, however, provided information primarily about backbone folding or frequently reflect side-chain conformation in an ill defined manner.

In the course of studies on the conformational changes in α lactalbumin,

* NSF Pre-doctoral Trainee

we have examined fluorescence properties of this protein from four different animal species where systematic replacement of tryptophan residues has occurred. These observations appear to substantiate the predictions made by the model of Browne et al. as to the arrangement of aromatic side chains and disulfide bridges in the region of the α-lactalbumin molecule, homologous with the active site cleft of lysozyme.

Experimental

Goat α lactalbumin (GAL) was prepared as described previously (16). Bovine α lactalbumin (BAL) was prepared by a modification of the method of Robbins and Kronman (17), where the final crystallization from ammonium sulfate was replaced by chromatography on Sephadex G-100. Human α lactalbumin (HAL) was prepared by a method of Findlay and Brew (18), while guinea-pig α lactalbumin (GPAL) was obtained by the method of Brew and Campbell (19). Fluorescence measurements were made as described earlier (16). Quantum yields (Q) were derived from corrected emission spectra and were calculated relative to tryptophan at neutral pH. In experiments where the yield was determined as a function of temperature, a so-called "Q_{app}" was obtained from the corrected emission spectra at the temperature in question, with the absorbance being measured at 25°. In this case also, a tryptophan reference at 25° was used in the calculation. Excitation was carried out at 295 nm, where only tryptophan residues are excited. Yields were calculated relative to the amino acid tryptophan (pH 7.00, 0.02M Tris; Q = 0.13, reference 20).

Results and Discussion

Interpretation of the fluorescence observations has been made on the basis of differences in tryptophan sequence homology for the four α lactalbumins (Table 1). The tryptophans of BAL and GAL occupy homologous positions, whereas in HAL, Trp 26 is replaced with leucine and in GPAL, Trp 60 is replaced with phenylalanine. Such a situation should make it possible to obtain information about the role of individual residues in determining fluorescence properties.

Table 1

Sequence Homology of Tryptophans in α Lactalbumin[a]

	Residue				Reference
	Trp 26	Trp 60	Trp 104	Trp 118	
BAL	+	+	+	+	1-4
GAL	+	+	+	+	b
HAL	-(Leu)	+	+	+	18
GPAL	+	-(Phe)	+	+	21

a. Numbering of residues is in terms of the BAL sequence. The homologous residues in lysozyme are 28, 64, 108 and 123. Where a tryptophan residue is missing, its replacement is indicated in parentheses.

b. K. Brew, unpublished observation.

Fluorescence parameters derived from emission spectra for all four proteins are summarized in Table 2 for the native protein (pH 8.00-8.50). The fluorescence parameters of BAL and GAL are very similar, which is not unexpected in view of the identity of the tryptophans in the sequences of BAL and GAL. The apparent fluorescence parameters for HAL are nearly identical with those of BAL and GAL, but in view of the difference in sequence homology (Table 1), this similarity must be regarded as more apparent than real (see reference 22 for further consideration of the fluorescence properties of HAL). The most striking observation (Table 2), however, is the marked difference between GPAL and the other three α lactalbumins. GPAL exhibits a greater than two-fold larger yield than BAL and GAL. This gross difference exists over the entire pH range 2 to 8.5 (22).

If the fractional absorption at the excitation wavelength is the same for all of the tryptophan residues in the protein molecule, then the observed

Table 2

Parameters of Tryptophan Fluorescence of α Lactalbumin

Protein	Q	λ_{max} (nm)
BAL	0.0328	330
GAL	0.0298	330
HAL	0.0314	330
GPAL	0.0738	329

a. Reproducibility of Q, ± 3%. Reproducibility of λ_{max}, ± 1 nm. Measurements made in 0.15M KCl, 25.0° in the pH range 8.00 to 8.50.

quantum yield is a number average yield. Thus, the observed yields for BAL and GAL are given by:

$$Q_{obs.} = \tfrac{1}{4} \left[\overline{Q}_{26} + Q_{60} + Q_{104} + Q_{118}\right] \quad (1)$$

Similar expressions can be written for the observed yields of HAL and GPAL. Thus, if we assume that all of the tryptophans in the α-lactalbumin molecule emit independently, we can calculate the yields of residues 26 and 60 from Equation 2 and 3, respectively.

$$Q_{26} = 4(Q_{GAL})_{obs.} - 3(Q_{HAL})_{obs.} \quad (2a)$$

$$Q_{26} = 4(Q_{BAL})_{obs.} - 3(Q_{HAL})_{obs.} \quad (2b)$$

$$Q_{60} = 4(Q_{GAL})_{obs.} - 3(Q_{GPAL})_{obs.} \quad (3a)$$

$$Q_{60} = 4(Q_{BAL})_{obs.} - 3(Q_{GPAL})_{obs.} \quad (3b)$$

The value of Q_{26} of ca. 0.02 to 0.03 obtained from such calculations is reasonable from a physical point of view; its significance will be considered elsewhere (22). However, no reasonable solution exists for Q_{60}; Q_{60} calculated

from Equation 3 is -0.09 to -0.11, a physically meaningless result. The fact that the yields of HAL, GAL and BAL (all containing Tryptophan 60) are markedly lower than that of GPAL (missing Tryptophan 60), suggests that the presence of Tryptophan 60 results in marked quenching of the fluorescence of one or more of the other tryptophans in the α-lactalbumin molecule.

This quenching appears to be a function of the three-dimensional folding of the α-lactalbumin molecule. Although the acid conformational change (23) does not "normalize" these residues; i.e., the marked difference in yield between HAL, BAL and GAL, and GPAL does not disappear at low pH (22), a thermally induced conformational change occurring with all four proteins appears to accomplish this. (See reference 22 for a detailed description of the thermally induced conformational change.) At temperatures above 40°, the emission spectra of the four proteins show a progressive long wavelength shift with spectra for GAL, BAL and HAL giving the largest shift under comparable conditions. In the case of GAL, BAL and HAL, the conformational transition is reflected as extrema in plots of fluorescence versus temperature at the longer wavelengths (i.e., 350-360 nm) and as inflections at the shorter wavelengths (i.e., 310 nm). The transition temperatures, $T_{\frac{1}{2}}$, derived from such curves were found to be essentially the same (52-55°) for GAL, BAL and HAL and independent of the wavelength of observation. The thermally induced conformational change observed with GPAL by contrast with GAL, BAL and HAL, does not show extrema in F-versus-T curves at the longer wavelengths, but exhibits an inflection at the lower wavelengths. The transition temperature for GPAL, calculated from the observations at the lower wavelengths (310 nm) in the emission spectrum, was about 65°. It thus seems reasonable to identify the changes in long wavelength emission seen for BAL, GAL and HAL with Trp 60, which is missing in GPAL; or perhaps even more likely with Trp 60 and whatever tryptophan residue or residues became quenched as a result of the presence of Trp 60 (see above). The latter conclusion is borne out by the observation that the apparent quantum yield for all four proteins approaches a common limit

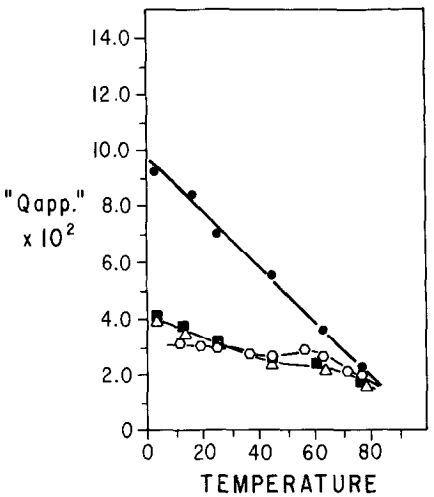

Fig. 1: Apparent Quantum Yields of α Lactalbumin as a Function of Temperature. Bovine, △ ; Goat, ⬭ ; Human, ■ ; Guinea Pig, ● .

at the upper end of the thermal transition (Figure 1). By 70-75°, conversion of all four proteins to high temperature conformers is essentially complete. As seen in Figure 1, the apparent yield of GPAL in this temperature range is only about 30% higher than that of the other three proteins, in contrast with the observations at much lower temperature where a difference of the order of two to three fold was seen. Such a difference leads to no difficulty in calculating a physically "reasonable" value for Q_{60} (see above). The high temperature conformer, it should be noted, is not the completely unfolded protein; i.e., the emission maxima for all four proteins at 76° range from 338 to 342 nm, indicating that some of the tryptophans in the protein molecule are still shielded from the aqueous environment by the three-dimensional folding of the protein molecule. The thermally induced conformational change appears to have changed the spatial relationship between Trp 60 and another tryptophan residue or residues, thereby destroying the interactions which lead to quenching of the fluorescence at the lower temperatures.

Examination of the "lysozyme analogy model" of Browne et. al (5) indicates that of the four tryptophans, only one, Trp 104, is sufficiently close to be able to interact likely with Trp 60. Trp 118 is quite distant from Trp 60, while

Trp 26 is about 9-10Å removed from 60. Since the yield of HAL, which is missing Trp 26, is comparable with that of GAL and BAL (Table 2), it is unlikely that this residue is the one which interacts with Trp 60. Trp 104, by contrast, lies within 7Å of Trp 60.

The most plausable explanation for the "interaction" of Trp 60 and 104 would seem to be non-radiative transfer of the excitation energy from Trp 104 to 60 with subsequent quenching of the emission from the latter residue. The model of Browne et al. (5) indicates that Trp 60 lies within 6Å of the 61-76 and 73-91 disulfide bridges. The quenching of tryptophan or indole fluorescence observed with sulfur-containing compounds (24-26) suggests that the close proximity of two such disulfide bridges to Trp 60 may result in marked quenching of its emission.

The critical transfer distance for Trp-Trp transfer depends upon the yield of the donor, the overlap of donor emission--and acceptor absorption--spectra, as well as on the mutual orientation of the donor and acceptor groups. Critical transfer distances of 7-9Å are calculated for the emission spectra of a tryptophan in a "typical" protein environment and for a random orientation of donor and acceptor (27). The efficiency of transfer from Trp 104 to Trp 60 in α lactalbumin, corresponding to such critical transfer distances, would be 50 to 80%. A favorable orientation would make the transfer still more efficient. The fact that the thermal transitions of α lactalbumin (Figure 1) obliterates the proposed interaction of Trp 60 and 104 without completely denaturing the molecule, suggests that such a preferential orientation of residues may indeed exist. The close proximity of Trp 60 and 104 and of disulfide bridges 61-76 and 73-91, predicted by the "lysozyme analogy model" of Browne et al. (5), would thus seem to account satisfactorily for the fluorescent properties of α lactalbumin described in this paper. These observations, therefore, lend further credence to the validity of the model, particularly with respect to the specification of side-chain geometry.

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