Biophysical Chemistry 11 (1980) 217–232

COMPARATIVE FLUORESCENCE PROPERTIES OF BOVINE, GOAT, HUMAN AND GUINEA PIG α LACTALBUMIN. CHARACTERIZATION OF THE ENVIRONMENTS OF INDIVIDUAL TRYPTOPHAN RESIDUES IN PARTIALLY FOLDED CONFORMERS ☆

Phillip B. SOMMERS * and Martin J. KRONMAN
Biochemistry Department, Upstate Medical Center, State University of New York, Syracuse, New York 13210, USA

Received 15 May 1979
Revised manuscript received 30 October 1979

α Lactalbumin exists as a partially folded conformer (U form) at acid pH. A second partially folded conformer (H form) is formed above 60°. Comparison of the changes in tryptophan fluorescence which occur on forming U and H for the bovine, goat, human and guinea pig proteins, as well as analysis of fluorescence properties for the bovine protein and an N bromo succinimide derivative of this protein, have made it possible to determine which tryptophan residues give rise to such changes in fluorescence, and to draw a distinction between the molecular structure of the U and H forms of the protein. Trp 28 and 109 in the native state transfer their excitation energy to trp 63 whose fluorescence is quenched by a pair of vicinal disulfide bridges. This process persists in the U form of the protein, but is absent in the H conformer. Most of the change in fluorescence seen in the N ⇌ U conversion is due to increase in yield of trp 28, while the changes in fluorescence occurring on formation of the H form are due to exposure of trp 63 and elimination of its quenching and/or excited state transfer from 28 to 109.

1. Introduction

Numerous studies have shown that the conformational change for α lactalbumin (the B protein of the lactose synthetase system, ref. [1]) which occurs at acid pH strongly effects the molecular environment of tryptophan residues [2,6]. The low pH form of the protein appears to be an intermediate in folding completely denature protein [7]. We report here studies of the comparative fluorescence properties of GAL ‡1, BAL, HAL and GPAL as a function of pH and temperature. GAL and BAL each have four tryptophan residues occupying homologous positions (28, 63, 108 and 123) ‡2 in the amino acid sequence. By contrast HAL and GPAL each have three such residues. The tryptophans of HAL are at positions 63, 108 and 123 with trp 28 being replaced by leu, while the three tryptophans of GPAL are at positions 28, 109 and 123 with trp 63 being replaced by phe [8]. Thus, by comparing the fluorescence properties of two α lactalbumins having three tryptophan residues with BAL and GAL having four such residues we have been able to draw conclusions as to which tryptophan residues may be involved in the acid conformational change and in a structural change induced thermally near neutral pH. A preliminary account of these observations has been given earlier [6].

☆ Paper XII in the series Inter- and Intramolecular Interactions of α Lactalbumin. The previous paper in the series is Kronman et al. [4].

* Predoctoral Trainee, National Science Foundation. Part of the research described here was included in a thesis submitted in a partial fulfillment of the requirements for the Ph.D. Degree, 1973.
Present address: Glass Columbia Medical Service Inc., 1475 Barnum Avenue, Bridgeport, Conn. 06610, USA.

‡1 Abbreviations used: GAL, goat α lacalbumin; BAL, bovine α lactalbumin; HAL, human α lactalbumin, GPAL, guinea pig lactalbumin.

‡2 Sequence positions are given in terms of the lysozyme amino acid sequence to facilitate discussion of the observations in terms of the "lysozyme analogy model" (see section 4.1.).

2. Experimental

2.1. Preparation of α lactalbumins

The procedure for preparing α lactalbumin (Procedure B) was substituted for our usual method (procedure A, ref. [9]) since it was found [5] that protein prepared by this manner gave more reproducible fluorescence parameters. This method is a modification of a procedure previously used for BAL [10]. The "crude globulin fraction" described in that procedure is further treated as follows: The "crude globulin" is dissolved in a minimum amount of distilled water, adjusted to pH 7.5 with 1M NH_4OH; stirred for about 30 minutes, and the precipitate separated by centrifugation and discarded. The supernatent solution is made 3M in ammonium sulfate, stirred for 30 minutes and the product (the precipitate) separated by low speed centrifugation at ambient temperature. The finnal purification is on a Biorad P-60 column (10 × 100 cm) using a pH 7.5, 0.05 ionic strength phosphate buffer. This procedure has the advantage of not exposing the GAL to pH values below 4.6, nor to elevated temperatures. The GAL fraction was exhaustively dialysed against distilled water and then lyophilized. GPAL and HAL, gifts of Dr. K. Brew, were prepared by the methods of Brew and Campbell [11] and of Findley and Brew [12] respectively. BAL was isolated by a modification of the method of Robbins and Kronman [9] and was chromatographed on a 10 × 100 cm column of G-100 in pH 7.5, 0.05 M phosphate buffer prior to dialysis and lyophilization.

2.2. Fluorescence measurements

Solutions of α lactalbumin for use in fluorescence measurements ($A_{295} < 0.1$) were made from a stock solution prepared daily by dissolving a suitable amount of lyophilized protein in 0.15 m KCl. The actual absorbance of dilutions was determined with a Cary 16 spectrophotometer thermostated at the temperature at which the fluorescence measurements were to be carried out. Prior to absorbance and fluorescence measurements all solutions were filtered through Millipore HA, Triton-free filters to remove dust and extraneous particulate matter. The pH of solutions was determined at ambient temperature using the expanded scale of a Radiometer Model 26 pH meter.

The instrument and techniques used for fluorescence measurements have been described earlier [4, 13]. Excitation was made at 295 nm. The cell contents were maintained at the desired temperature using a thermostatted cell compartment and monitored continuously with a thermister probe integral with the cell cap. Cell contents were stirred continuously by a magnetic stirrer integral with the sample compartment.

Fluorescence data was computer analysed by methods described earlier [13]. A value of 0.13 was used for the quantum yield of tryptophan at neutral pH [14].

Spectral quantum yields, (SQY), [15], were computed from the corrected emission spectrum and the quantum yield using the relationship:

$$(\mathrm{SQY})_\lambda = (F_\lambda / A_P) Q_P \,, \qquad (1)$$

A_P is the area under the corrected emission spectrum. The computed values of Q and the absolute corrected emission spectra (SQY versus λ) were stored on magnetic tape for further computations, e.g. calculation of difference spectra (Δ SQY versus λ).

2.3. Measurement of fluorescence as a function of temperature

In order to circumvent the need to measure the absorbance of protein solutions at every temperature at which emission spectra were obtained, "apparent" quantum yields were calculated using protein emission spectral data at such temperatures together with absorbances for protein and tryptophan standard measured at 25°. Such "apparent" quantum yields were calculated relative to the standard measured at 25°. The long term stability of the spectrofluorimeter and its ability to compensate for changes in light source intensity made this procedure feasible.

2.4. Measurement of iodide quenching of tryptophan fluorescence

Iodide quenching of the tryptophan fluorescence of α lactalbumins was carried out by a procedure similar to that employed by Lehrer [16]. Aliquots of protein, KI and KCl stock solutions were mixed to give appropriate concentrations of I^- and α lactalbumin (ca. 0.1–0.15 mg/ml) and an ionic strength of 0.25 (KCl + KI). All such dilutions were 0.1 mM in sodium

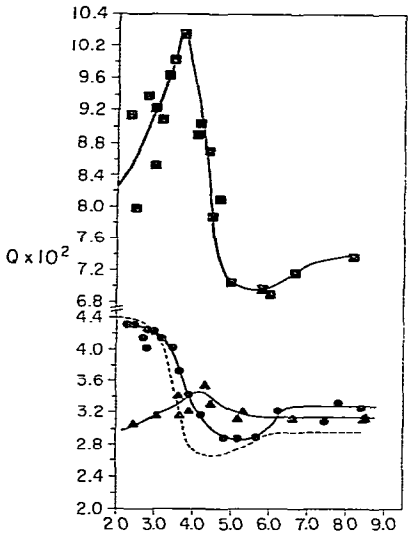


Fig. 1. The pH dependence of the quantum yield for tryptophan fluorescence at 25.0° in 0.15 M KCl for GAL (– – –); BAL, •: HAL, ▲ and GPAL, ■. The curve for GAL was determined by more than 60 data points which have been omitted in the interests of clarity. These yields were obtained from emission spectra comparable to those shown in figs. 2 and 3.

thiosulfate. Emission spectra were scanned for individual solutions containing iodide and their quantum yields calculated relative to that for an identical protein solution containing no quencher.

3. Results

3.1. Comparison of the pH dependence of the quantum yield

All four α lactalbumins exhibit marked changes in tryptophan fluorescence in going from neutral to acid pH (figs. 1–3). The pH dependencies of Q for BAL and GAL, which both have four tryptophans occupying the same positions in the amino acid sequence, are very similar; both show a monotonic increase to attain nearly identical values as the pH approached 2. The spectral transition curve for BAL, however, appeared to be shifted toward higher pH values relative to that for GAL. The pH dependencies of Q for HAL and for

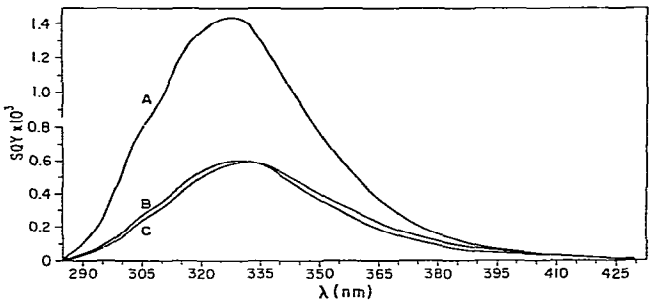


Fig. 2. Tryptophan emission spectra for GPAL (curve A), HAL (curve B) and BAL (curve C) at pH 6.5. The excitation wavelength was 295 nm. The other conditions are given in fig. 1.

GPAL (each having only three tryptophans) are strikingly different from those observed with GAL and BAL, in that the former proteins show an increase, followed or paralleled by a decrease in yield with decreasing pH. All of the α lactalbumins except HAL exhibited a small increase in Q going from about pH 4.5 to 5 toward alkaline pH. The magnitudes of these changes, however, are somewhat different (GPAL, 6%; BAL, 15%, GAL, 7%).

The most striking observation is that the yield for GPAL is about three fold greater than observed for GAL, BAL and HAL over the entire pH range. The origin of this difference will be considered in sect. 4.

3.2. The emission spectra of the four α lactalbumins

All four α lactalbumins have very similar emission spectra at neutral pH (fig. 2, table 1), with virtually

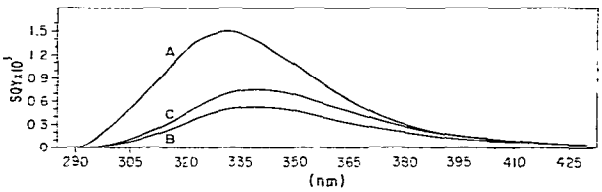


Fig. 3. Tryptophan emission spectra for GPAL (curve A), HAL (curve B) and BAL (curve C) at pH 2.5. The other conditions are given in fig. 2.

Table 1
Selected fluorescence parameters for the four α lactalbumin species a)

pH	Emission maximum, nm				Quantum yield $\times 10^2$			
	GAL	BAL	HAL	GPAL	GAL	BAL	HAL	GPAL
6.5	330	331	330	329	2.9	3.1	3.1	7.1
5.5	330	331	330	329	2.8	2.9	3.1	7.0
4.5	330	331	332	329	2.7	2.9	3.3	8.0
3.5	336	337	338	332	4.2	4.2	3.2	10.3
2.5	340	340	338	332	3.9	4.1	3.0	8.6

a) Experimental conditions as in fig. 1.

identical maxima and a characteristic inflection between 305 and 310 nm which cannot be due to tyrosine fluorescence since excitation was at 295 nm.

The spectra for the four proteins show considerable difference in shape near pH 2.5, where the protein should exist primarily in the U form [4] (fig. 3, table 1). BAL and GAL have similar spectral shapes and nearly identical emission maxima (table 1), a 9 to 10 nm shift being observed in the N ⇌ U conversion. While GPAL exhibits a 47% increase in yield in the N ⇌ U conversion, comparable with the change observed with BAL and GAL, the shift in emission maximum is only about 4 nm. HAL, by contrast, exhibits a rather small change in yield but shows an 8 nm shift during the same process. The difference spectrum (fig. 4) for the pH interval 2.48–6.25 for BAL is bimodal with positive and negative components comparable with what was found with GAL (data not shown). We have shown that the spectral change for GAL down to ca. 337 nm reflects only the N ⇌ U transition [5]. The negative component corresponds, in part, to a superimposed pH dependent quenching process.

The difference spectra obtained with GPAL and with HAL likewise reflect superposition of a second pH dependent process on the N ⇌ U transition. The pH 3.82–6.66 spectrum (curve C, fig. 4) for GPAL and the pH 2.47–6.63 spectrum for HAL (curve B, fig. 4) both exhibit the bimodal form found with BAL and GAL with different relative amplitudes for the positive and negative components. The superimposed quenching seen with these two proteins as a decrease in Q with decreasing pH (fig. 1) is reflected also in characteristic difference spectra with only positive

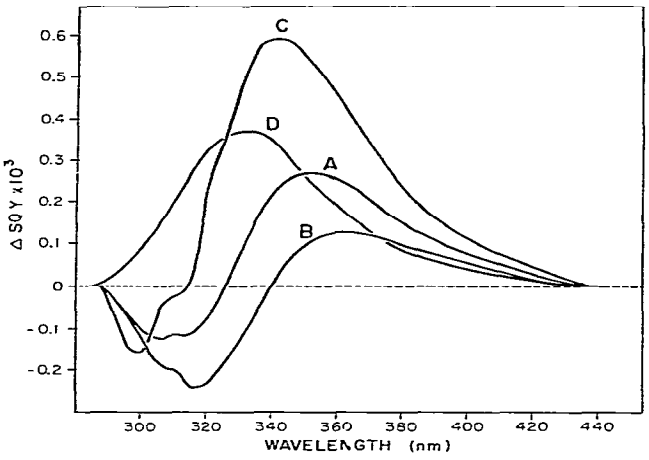


Fig. 4. Computed tryptophan difference spectra at 25.0° for BAL, pH 2.48–6.25 (curve A); HAL, pH 2.47–6.63 (curve B); GPAL, pH 3.82–666 (curve C); GPAL, pH 3.82–2.49 (curve D). These spectra were calculated from absolute spectra similar to those given in figs. 2 and 3.

components (curve C, fig. 4). In the case of GPAL there is a fluorescent component whose emission is centered at about 330 nm reflecting quenching in the pH region 2.49 to 3.82 (compare with the corresponding quantum yield curve, fig. 1). The HAL fluorescence quenched in roughly the same pH range gives rise to a rather small spectral component centered at about 350 nm (data not shown).

3.3. The effect of temperature on α lactalbumin fluorescence

The thermal transition of α lactalbumin seen by Barel [17] using optical rotation dispersion has been examined here using fluorescence measurements. The progress of the thermally induced transition is most strikingly seen in plots of fluorescence intensity versus temperature (figs. 5 to 8). The fluorescence at longer wavelengths, e.g. 350, 360 nm (fig. 5) shows "thermal quenching" of the low temperature conformer initially, an increase in intensity in the transition region and finally, a further decrease in intensity reflecting "thermal quenching" of the fluorescence of the high temperature conformer. The conformational transition is somewhat less obvious from data obtained at lower

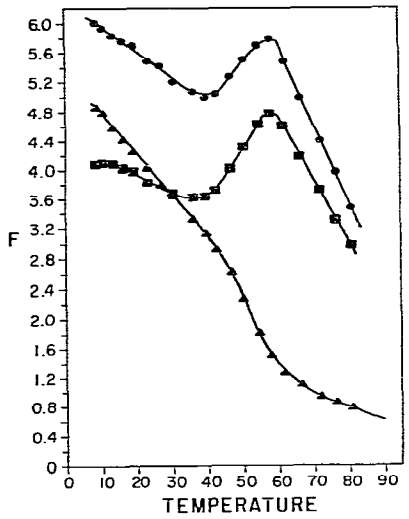


Fig. 5. Temperature dependence of the fluorescent intensity for BAL at 360 (■), 350 (●) and 310 (▲) nm at pH 6.0 ± 0.1 in 0.15 M KCl. The excitation wavelength was 295 nm.

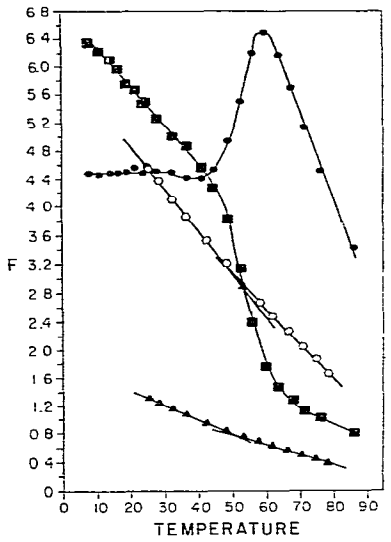


Fig. 7. Temperature dependence of the fluorescence intensity for GAL at pH 6.86 (350 nm, ●; 310 nm, ■) and at pH 2.48 (350 nm, ○; 310 nm, ▲). The other conditions are given in fig. 5.

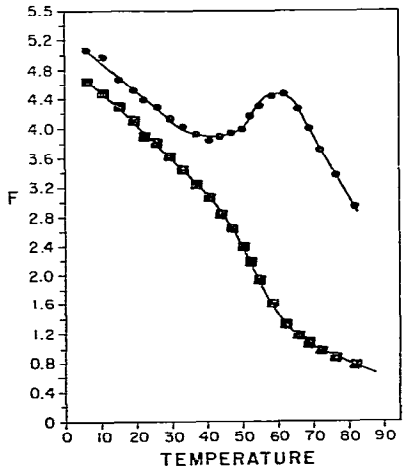


Fig. 6. Temperature dependence of the fluorescence intensity for HAL at 350 (●) and 310 (■) nm. The other conditions were as in fig. 5.

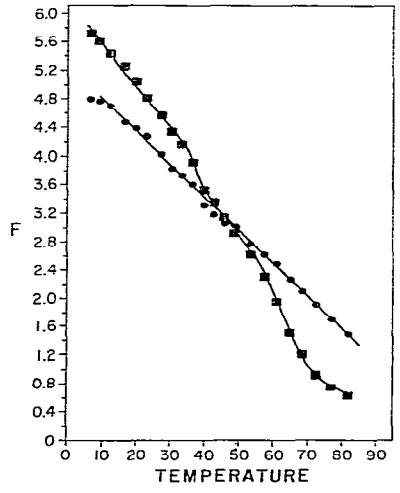


Fig. 8. Temperature dependence of the fluorescence intensity for GPAL at pH 6.80 at 350 (●) and 310 (■) nm. The other conditions are given in fig. 5.

wavelength, e.g. 310 nm (fig. 5), since the change is a *decrease* in fluorescence intensity which is superimposed on the usual thermal quenching. The shapes of the curves of fluorescence intensity versus temperature are almost identical for HAL and BAL at comparable wavelengths in the emission spectrum (compare figs. 6 and 7). While the changes with GAL are qualitatively comparable with those seen with HAL and BAL (compare figs. 5 and 9), there is little if any dependence of F on temperature for GAL below 40°.

It is noteworthy that the low pH form, does not undergo the thermal transition (compare curves for pH 2.48 and 6.86, fig. 7), the fluorescence decreasing monotonically with increase in temperature. Furthermore, there is no dependence of Q on pH for GAL at 60° in contrast with obsrevations at 25°.

GPAL gives no indication of the thermally induced conformational change at longer wavelengths in the emission spectrum (fig. 8); the decrease in F is nearly linear at 350 nm. At lower wavelengths, however, such curves show inflections comparable with those seen with BAL, HAL and GAL (figs. 5–8) from which we conclude that the thermally induced conformational change *does* occur for GPAL, but does not give rise to changes in fluorescence at the longer wavelengths.

Heating of solutions of protein to temperatures *up to ca.* 60° was reversible, i.e. the 25° spectra were regained in heating and cooling cycles. By 80°, however, marked irreversible changes had occurred for all four α lactalbumins. Since the thermally induced conformational change is essentially complete near 60° (see below), the lack of reversibility from the higher temperature is most likely due to secondary processes.

Conversion of α lactalbumin to the high temperature conformer brings the fluorescence properties of

Table 2
Comparison of fluorescence properties of the high temperature conformer of α lactalbumin at 77° a)

Protein	Q_{app}	Emission maximum, nm
BAL	0.0164	343
GAL	0.0192	343
HAL	0.0184	342
GPAL	0.0222	337

a) Experimental conditions as in fig. 1. The uncertainty in temperature is ±0.6°, and in emission maximum, ±1 nm.

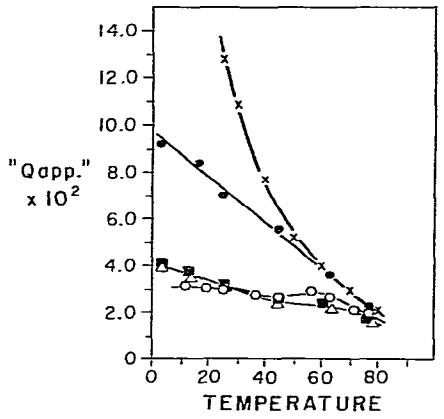


Fig. 9. Temperature dependence of the apparent quantum yield (see Materials and Methods) for tryptophan fluorescence for GPAL (•), HAL (■), BAL (△), GAL (○) and free tryptophan (×). The solvent for protein was pH 6.0 ± 0.1, 0.15 M KCl. The tryptophan was dissolved in water and adjusted to pH 6.0 at 25.

GAL, BAL and HAL more nearly into correspondence with those of GPAL (fig. 9). While the quantum yield of GPAL at 25° is nearly 3 fold greater than for BAL, GAL and HAL, over the entire pH range (fig. 1), the difference between them at 77° is, at most, about 35% (table 2). The emission maxima at 77° for all four proteins is significantly different from 350 nm, the value expected for a completely unfolded protein (table 2).

3.4. The thermal transition temperature

The fluorescence observed at a given wavelength, λ, for a given temperature is

$$(F_\lambda)_{obs} = (1 - \alpha_\lambda)(F_\lambda)_{N,T} + \alpha(F_\lambda)_{H,T}\,, \qquad (2)$$

where the subscripts N and H refer to the native and „high" temperature conformers, α is the fraction of conversion of the N to the H form and $(F_\lambda)_{N,T}$ and $(F_\lambda)_{H,T}$ represent the intrinsic fluorescence at a wavelength of the two conformers *at the temperature of* the measurement. In order to determine α we have assumed that $(F_\lambda)_{N,H}$ and $(F_\lambda)_{H,T}$ are linear functions of temperature. Inspection of figs. 6–9 indicates that with the possible exception of GAL this is a reasonable approximation. $(F_\lambda)_{N,T}$ and $(F_\lambda)_{H,T}$ were thus

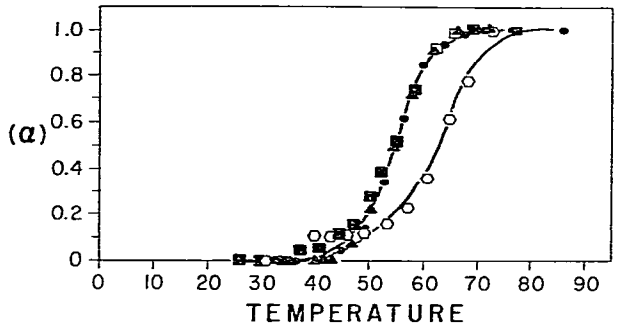


Fig. 10. The temperature dependence of the parameter α (equation 4) for the thermal transition for BAL (▲), HAL (■), GAL (●) and GPAL (○). α was calculated from data at 310 nm for GPAL and at 350 nm for the others.

obtained by extrapolation of the linear regions of the F versus T curves.

The thermal transitions curves (fig. 10) are rather sharp, the change taking place over a 20° temperature range. The transition temperatures, $T_{1/2}$ (table 3) are nearly the same for HAL, BAL and GAL and independent of the wavelength of observation. Values obtained for the transition temperatures for BAL, HAL and GPAL are in good agreement with those observed respectively by Barel et al. [17] and by Takase et al. [19].

Van't Hoff plots of the thermal data were linear for GAL, GAL and HAL up to about 60° with some indication of curvature at higher temperature. Curvature was seen for GPAL even below 60°. Values of ΔH° calculated from the linear portion of the Van't Hoff plots were 45 ± 4 (HAL), 59 ± 4 (BAL) and 67 ± 3 (GAL) kcal/mole. These are to be compared with values of 55 and 50 kcal obtained by Barel et al. [17] for HAL and BAL respectively.

Table 3
Transition temperatures for the thermally induced conformational change of α lactalbumin [a]

	$T_{1/2}$, deg.		
Protein	310 nm	350 nm	Literature value
BAL	55	51	50 [b], 53 [c]
GAL	55	55	–
HAL	55	55	56 [c]
GPAL	63	–	62 [c]

a) The transition temperature, $T_{1/2}$, is the temperature at which α equals 0.5 (fig. 10).
b) Barel et al. [17], optical rotation dispersion measurements.
c) Takase et al. [19], ultraviolet absorption difference spectral measurements.

3.5. Exposure of tryptophans in the high temperature conformer

In order to determine if the high temperature conformer of α lactalbumin might be an unfolded form of the protein with more tryptophans exposed to solvent than the N form of the protein (25°, near neutral pH) we carried out iodide quenching measurements at 25 and at 71.6° (GAL, HAL) and 74° (GPAL). The

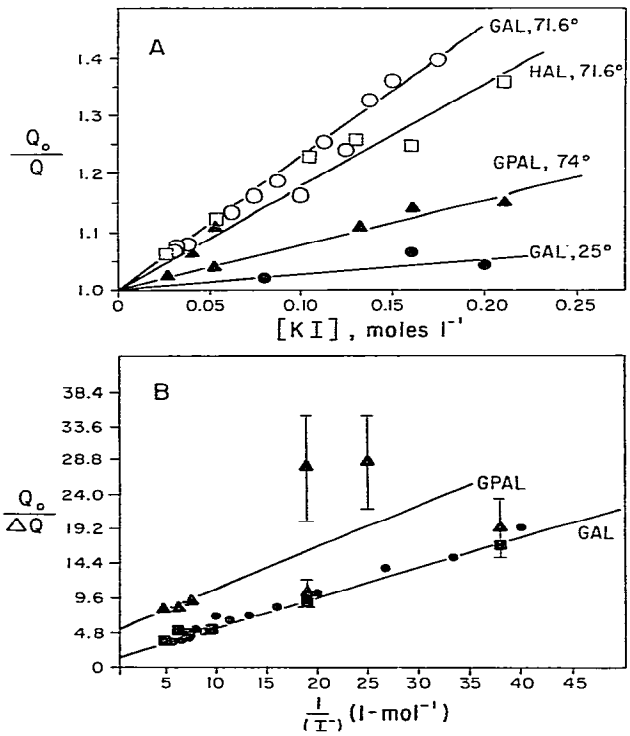


Fig. 11. Iodide quenching of tryptophan fluorescence for BAL, HAL and GPAL at pH 6.6. A. Stern Volmer plot (eq. (5)). B. Modified Stern–Volmer plot (eq. (7)). The excitation wavelength was 295 nm.

Table 4
Stern–Volmer and modified Stern–Volmer parameters of model compounds and the four α lactalbumin species at elevated temperature [a]

Substance	Stern–Volmer plot	Modified Stern–Volmer plot	
(1)	K_q [b] (2)	f_e (3)	$(K_q)_e$ (4)
Tryptophan	5.0 (12.0)	–	–
N acetyl tryptophan ethyl ester	2.8 (5.2)	–	–
GAL	2.31±0.003	0.82±1.15	2.7± 0.7
HAL	1.38±0.008	0.49±0.05	5.3±0.6
GPAL	0.60±0.003	0.2	8

a) See fig. 11 for experimental details.
b) Values in parentheses are for 25°C. The quenching constants observed for GAL, HAL and GPAL at 25° were less than 0.3. Since the precision of all the measurements at 25° was not high, no attempt was made to carry out an analysis using the modified Stern–Volmer equation.

latter temperatures are sufficiently high so insure complete transformation to the high temperature form of the protein, but sufficiently low to avoid irreversible secondary processes (see above).

Analysis of the fluorescence quenching data was done with the Stern–Volmer equation:

$$F_0/F = 1 + K_q(X)\,, \tag{3}$$

where F and F_0 are the fluorescent intensities or quantum yields in the presence and absence of quencher. The parameters obtained from Stern–Volmer plots (fig. 11A), for BAL, HAL, GPAL tryptophan and N acetyl tryptophan ethyl ester are summarized in table 4. K_q values for the latter two substances are in good agreement with those obtained by Lehrer [16] at 25°. K_q for iodide quenching of indole derivatives is given by [16]:

$$\frac{(K_q)_T}{(K_q)_{298}} = \frac{(F_0)_T(T/\eta_T)}{(F_0)_{298}(298/\eta_{298})}\,, \tag{4}$$

where $(F_0)_{298}$ are the fluorescence intensities in the absence of quencher at absolute temperatures T and 298° and η_{298} and η_T are the corresponding viscosities. The value of K_q, calculated for 71.6° from the measured value at 25.0° was 4.8 in good agreement with the value of 5.0 obtained experimentally (column 2, table 4).

Iodide quenching of tryptophan fluorescence of GAL at 52 ° was very weak (fig. 11A); the value of K_q obtained at this temperature was only about 6% of that obtained with the free amino acid. Quenching data for HAL and GPAL at 25° was similar to that observed with GAL. The conformer of GAL which exists at 71.6°, however, shows marked quenching by iodide. K_q is only 20% lower than that observed with N acetyl tryptophan ethyl ester (column 2, table 4). Iodide quenching of tryptophan fluorescence of the high temperature conformer of GPAL is very weak by contrast; K_q is almost an order of magnitude lower than the value observed for the free amino acid at the same temperature. Quenching observed with HAL at 71.6° is somewhat smaller than seen with GAL, but considerably greater than found with GPAL (fig. 11A).

Values of K_q obtained in the conventional Stern–Volmer analysis of quenching data for proteins reflect accessibility of tryptophan residues, as well as the relative magnitude of their contribution to the quantum yield. Lehrer [16] has developed a modified form of the Stern–Volmer equation which, to some degree, resolves this ambiguity:

$$\left(\frac{F_0}{\Delta F}\right)_{x\to 0} = \frac{1}{(x)\Sigma_i f_i(K_q)_i} + \frac{\Sigma_i f_i(K_q)_i^2}{\Sigma_i f_i(K_q)_i}\,, \tag{5}$$

where ΔF is the change in fluorescence in presence of quencher at concentration (x), $(K_q)_i$ is the quenching constant for the ith tryptophan and f_i is the fractional contribution for such a tryptophan residue to the measured fluorescence. The sum is taken over all tryptophan residues. The reciprocal of the intercept of this equation can be regarded as an "effective" fractional contribution, f_{eff}, while the ratio of the intercept to the slope can be seen as an effective quenching constant, $(K_q)_{eff}$.

The modified Stern–Volmer analysis for GAL (fig. 11B, columns 3, 4, table 4) gives values of 0.82 ± 0.15 and 2.7 ± 0.7 for f_{eff} and $(K_q)_{eff}$ respectively. Thus, it appears that most of the tryptophan fluorescence of GAL in the high temperature conformer can be quenched with an efficiency comparable to that observed with the free amino acid. The least squares fit of the HAL data in the form of eq. (5) yields a value of f_{eff} which is somewhat lower than that found for GAL (column 3, table 4) and an effective quenching constant comparable with that found for the free

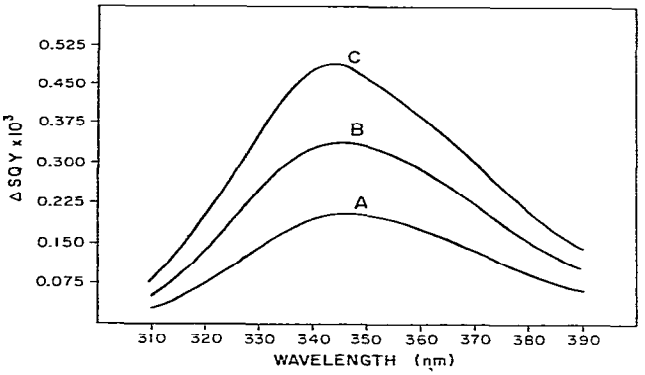


Fig. 12. Computed difference spectra for iodide quenched GAL. The difference spectra were calculated relative to a spectrum obtained for a comparable protein solution containing no KI. The other conditions are given in fig. 11. Curve A, 0.0625 M KI; Curve B, 0.113 M KI; Curve C, 0.175 M KI.

amino acid. The degree of quenching of tryptophan fluorescence for GPAL is too small to yield precise values of the quenching parameters; the best line through the data points in the modified Stern—volmer plot, however, (fig. 11B), yields values of 0.2 and 8 for f_{eff} and $(K_q)_c$ respectively, indicating that fluorescence of this protein cannot be iodide quenched to the degree found for HAL and GAL.

The difference spectra (fig. 12) of I^- quenched residues have the form of absolute spectra of tryptophans with maxima close to 345 nm, slightly lower than observed with the free amino acid in water.

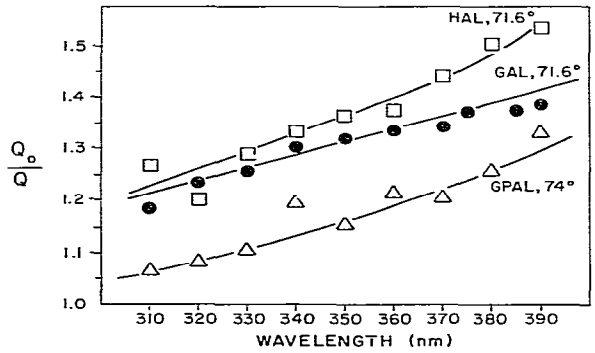


Fig. 13. Dependence of iodide quenching of tryptophan fluorescence on the wavelength of observation in the emission spectrum. The KI concentration was 0.21 M. The other experimental conditions are given in fig. 11.

The fact that emission maxima for BAL, GAL, HAL and GPAL lie below 350 nm at 77° (table 2) indicates that complete unfolding of the protein has not occurred during the thermally induced conformational change. Furtner evidence for this conclusion is seen in plots of F_0/F versus wavelength of observation in the emission spectrum (fig. 13). Since all of the tryptophan residues in a completely unfolded protein should have comparable emission properties, the fact of a significant dependence on wavelength of observations suggests that sufficient structure remains in the high temperature conformer of α lactalbumin to produce different environments for tryptophans.

4. Discussion

4.1. The three dimensional structure of α lactalbumin

Discussion of the changes in the molecular structure of α lactalbumin at low pH and at elevated temperature should ideally be made in the context of an X-ray crystallographic structure for the native protein. No such structure, however, has been reported as yet. Browne et al. [21] proposed that the backbone folding of α lactalbumin and hen's egg white lysozyme were very similar, owing to the fact of a high degree of amino acid sequence homology. Evidence for structural similarity has been summarized in Paper X in this series [22] and in the paper by Warme et al. [21]. The structure calculated for α lactalbumin by an energy minimization procedure [21] was comparable to that proposed by Browne et al. with the exception of the "tail" region of the molecule. Warme et al. [21] showed that this "tail" region containing trp123 could exist in any of three energetically equivalent conformations, T2, T3 and T4. In "tail" conformation T2 trp 123 is on the surface of the molecule with one side of the indole ring in contact with solvent, while in the other two conformations, "tail" residues form a hydrophobic pocket surrounding trp 123. The other three tryptophans (28, 63 and 108) are located in a cleftlike region of the molecule (see fig. 13, ref. [22] for a drawing of the overall shape of the molecule based on a molecular model constructed according to Browne et al.). We shall refer subsequently to the molecular environments of these tryptophans as deduced from the model.

4.2. The low quantum yield of BAL, HAL, and GAL

BAL, GAL and HAL have the lowest quantum yield of tryptophan fluorescence of the group of 17 proteins studied by Kronman and Holmes [13]. We have shown here that the large difference in yield between GPAL and the other α lactalbumins is largely abolished during the thermally induced conformational change which the four proteins undergo (fig. 9, tables 1 and 2), but not during the acid conformational change (fig. 1). The radical difference in yield between GPAL and the three other α lactalbumins must therefore, be the result of a characteristic spatial relationship of tryptophan residues in the protein molecule. If we assume additivity of emission from all four tryptophans the quantum yield of trp 63 can be obtained from:

$$Q_{63} = 4Q_{GAL} - 3Q_{GPAL} ,$$

or

$$Q_{63} = 4Q_{BAL} - 3Q_{GPAL} . \quad (6)$$

Such a calculation results in negative values of Q_{63}. This result indicates that the assumption of independent emission from the four tryptophans implicit in the above calculation is not valid. The manner by which the presence of trp 63 in BAL, GAL and HAL gives rise to low yields is discussed below. Although the poorer precision of the lower values of Q at higher temperature and the use of "apparent" yields renders the data subject to some uncertainty, there is little doubt that the thermally induced conformational change results in nearly independent emission from the four tryptophans, even though the high temperature conformer retains organized structure (see below).

We propose that energy transfer between tryptophans is singificant in α lactalbumins. The efficiency, e, of such transfer from donor to acceptor tryptophans will be given by the Forster equation:

$$e = r^{-6}/(r^{-6} + R_0^{-6}) , \quad (7)$$

where r is the distance between the two tryptophans and R_0, the so-called critical transfer distance is determined by the extent of overlap of emission and absorption spectra of acceptor and donor troups respectively and by their mutual orientation [24]. Eisinger and Lamola [24] calculate R_0 to be 6.9 to 8.7 Å for inter-tryptophan transfer.

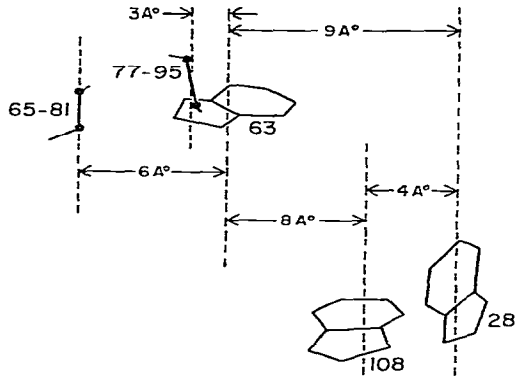


Fig. 14. Distances between trp 28, 109, 63 and disulfide gridges 77–95 and 65–81 as obtained from a molecular model built by the procedure of Browne et al. [21].

Evaluation of transfer efficiencies using eq. (7) requires a knowledge of the inter-tryptophan distances, as well as their mutual orientation. In the absence fo requisite X-ray crystallographic data we have made use of distances derived from the "lysozyme analogy" model (fig. 14) (see above). Trp 123 is 15 to 25 Å from the other three, much too large for significant transfer. By contrast the trp 109–trp 28 distance is about 4 Å which would result in 100% transfer, while for trp 63–109 and trp 63–28 the transfer efficiencies would be in the range of 20 to 60 % (R_0 = 6.9–8.7 Å, ref. [24]).

We propose that the energy of excitation is channeled from trp 28 and 109 to trp 63 where it is quenched as a result of the close proximity of the latter residue to the disulphide bridges 77–95 and 65–81. Model compound studies have shown that the fluorescence of indole and tryptophan derivatives are quenched by sulfer containing compounds [25,27]. Our further discussion concerning the environment of tryptophans in native α lactalbumin and in its low pH and high temperature conformers will invoke this model of tryptophan quenching.

4.3. Quantum yields of individual tryptophans in BAL and GAL

We have estimated the yields of individual tryptophans by the method used to evaluate that of trp 63

Table 5
Quantum yields of individual tryptophans in goat and bovine α lactalbumin

Tryptophan residue	Q a)
28	0.02 d)
28	0.023 c); 0.031 b)
63 + 108	0.032–0.058 e)
123	0.054 d)

a) Yields of individual residues calculated as described in the text.
b) Calculated from data for GAL at pH 6.5 in the present study (table 1).
c) Calculated from data for BAL at pH 6.5 in the present study (table 1).
d) Calculated from the quantum yield data of Bell et al. [28] pH 7 (see text.
e) Calculated with a combination of data from Bell et al. [28] and from data of the present study. The range of values reflect differences in the yield for BAL and GAL.

(section 4.2). The value obtained for trp 28 is given in the second line of table 5.

An independent procedure similar to that employed by Imoto et al. for lysozyme [29] was also employed for calculating Q_{28} making use of quantum yield data obtained by Bell et al. [28] for native BAL and for derivatives of this protein which had been modified with N-bromo succinimide. Bell et al. [28] showed that trp 28 and 123 of BAL are the only residues modified by N-bromo succinimide, with reaction at trp 123 occurring *prior* to modification of trp 28. Two such derivatives were prepared; the first, $(BAL)_{0.95}$, had 0.95 moles of trp modified per mole of protein, corresponding to 95% reaction at trp 123, while the second, $(BAL)_{1.6}$, contained 1 mole of modified trp 123 and 0.6 moles of modified trp 28. Our calculations involved two steps: a) calculation of Q_{123} using the yields for $(BAL)_{0.95}$ and for native BAL (line 4, table 5); and b) using Q_{123} and yields for native BAL and $(BAL)_{1.6}$ we obtained Q_{28}. This value of Q_{28} is in excellent agreement with that calculated from HAL and BAL quantum yield data (compare lines 1 and 2, table 5). The fact that the quantum yield data which entered into these two sets of calculations were derived from completely different kinds of experiments lends confidence to the validity of the calculation.

The tabulated values of Q_{28} and Q_{123} were used to make an order of magnitude calculation of Q_{63}. Trp 28 and 108 are sufficiently close together (fig. 14) such that transfer efficiency between them may be of the order of 100%, resulting in comparable apparent yields for these two residues. Utilizing the value in table 5 for Q_{28} and for $(Q_{63} + Q_{108})$ we find Q_{63} to be of 0 to 0.01, i.e. trp 63 is highly quenched as predicted by the model outlined in the previous section.

Trp 123 appears to account for nearly half of the tryptophan fluorescence from BAL and GAL, while trp 28 and 108 each contribute about 20% (table 5). It should be emphasized that the values of the yield for trp and 108 refer to direct contribution to the emission in BAL and GAL with a high percentage of the excitation energy having been transferred to trp 63 and "lost" by quenching. If we assume that Q_{123} is the same in GPAL as in BAL and GAL, a value of 0.16 is found for $(Q_{28} + Q_{108})$ in GPAL. Comparison of this value with $(Q_{28} + Q_{108})$, ca. 2×0.023, obtained for the same residues in BAL, indicates that the transfer efficiency from 28 and 108 to 63 in BAL and GAL may be as high as 70%.

4.4. Molecular environment of tryptophans in the N state

Prior to considering the molecular changes which occur during the thermally induced structural change in α lactalbumin and in the $N \rightleftharpoons U$ transition, we will examine what is known of the environment of these residues in the context of the observations made in this study. Solvent perturbation measurements for BAL [2,13,19] show from 1.4 to 1.8 group exposed in the N state with about 0.4 groups less in GPAL [19], a marginal difference in terms of experimental error. Exposure is essentially identical for BAL, GAL and HAL. In contrast with the solvent perturbation observations, quenching by I^- is quite weak (fig. 11A, footnote, table 4). This difference is due to the fact that "long range" perturbants "see" chromophores somewhat below the surface of the protein molecule (see refs. 30;31] for discussion of properties of perturbants), while quenching by I^- ion is a dynamic process requiring a kinetic encounter with the fluorophore. We conclude from the solvent perturbation and I^- quenching observations that none of the four tryptophans are fully exposed; such residues are either buried or make

fractional contributions to the perturbation spectral shift.

With the exception of the uncertainty in the environment of trp 123, reflecting alternative conformations for the tail region of the molecule (see above), both the "lysozyme analogy" model and the structure of Warme et al. [23] indicate that trp 28, 63 and 108 are "buried" to varying degrees. Trp 28 is located in a hydrophobic pocket while trp 63 and 108 are located in the cleft region with the latter partially shielded from solvent and the former almost completely so by tyr 107, asp 102 and disulfide bridge 77–95 (fig. 14). We shall consider the environment of trp 123 at greater length below.

Our conclusion that none of the four tryptophans of BAL and GAL are completely exposed is in conflict with conclusions reached by other investigators. Bell et al. [28] claim that 123, which accounts for about 50% of the fluorescence of BAL (table 5), is in contact with the aqueous medium. They observed that the excitation spectrum of unmodified BAL was red shifted relative to that of the N bromo succinimide derivative of BAL, $(BAL)_{0.95}$ (see above, section 4.3) and conclude that "this red shift indicates that trp 118 (trp 123) is in a more exposed environment than the others". The emission maximum of such a water contacted tryptophan should lie close to 350 nm, some 25 nm higher than that they observed for BAL. If trp 123 were in contact with water as proposed by Bell et al. [28], a substantial *short* wavelength shift of emission spectrum should have been seen when this residue was modified with N bromo succinimide, contrary to what was observed. Their interpretation of the relative positions of the absorption spectra for buried and water contacted groups runs contrary to the well established dictum that the absorption spectrum of a water contacted tryptophan is blue shifted relative to one located in a non-polar region of a protein molecule (see ref. [30] for pertinent literature citations).

The correct interpretation of the observations of Bell et al. [28] leads to the conclusion that trp 123 is *not* exposed to solvent. Thus, while interconversion of the three "tail" conformations (see above) might take place under some conditions (see below), our observations and those of Bell et al. [28] show that in the unperturbed state the α lactalbumin molecule is most likely in conformation T3 or T4 with trp 123 buried in a hydrophobic pocket.

A second potential conflict with our conclusion that none of the tryptophans of α lactalbumin are completely exposed to solvent comes from the observations of Robbins and Holmes [32] who showed that a single tryptophan in BAL can form a charge transfer complex with N methyl nicotinamide. Formation of such a comples requires that the indole ring of the tryptophan side chain be capable of a face to face interaction with the pyridinium ring of the nicotinamide derivative. We propose that trp 123 forms such a complex and this occurs by altering the "tail" conformation from T3 to T4 or T2, where the tryptophan is in contact with water. This seems plausible in view of the comparable energies of the three conformations.

4.5. The thermally induced structural change

Barel et al. [17] reported that the thermally induced conformational change, which they refer to as "denaturation", occurs for BAL and HAL evidenced by changes in optical rotation at 364 nm. It is clear that the process that we report in the present paper is the same seen by Barel et al., since the thermodynamic parameters which characterize the conformational change are in good agreement (table 3).

Takase et al. [19] have measured tryptophan absorption difference spectra to characterize the temperature dependent conformational change. The maximum values of $\Delta\epsilon_{293}$ were −1300, −2100 and −2400 for HAL, GPAL and BAL respectively. Assuming additivity of absorbances we obtain from their data $\Delta\epsilon_{28} = -1100$ and $\Delta\epsilon_{63} = -300$. Since the sum of $\Delta\epsilon_{28}$ and $\Delta\epsilon_{63}$ is only −1400, $(\Delta\epsilon_{108} + \Delta\epsilon_{123})$ is equal to −100. It thus appears that the environment of trp 108 and/or 123 is altered in addition to that of trp and to some degree trp 63.

Although the magnitude of $\Delta\epsilon_{63}$ as calculated above is rather small, sample evidence is available in the present study to implicate trp 62 in the structural change:

1. The characteristic temperature dependence of the fluorescence seen with BAL, GAL and HAL is absent in GPAL (compare figs. 5–8).

2. The quantum yield anomaly seen at 25° on comparing values obtained for GPAL with those of BAL, GAL and HAL disappears at elevated temperature (compare values of Q in tables 1 and 2). The disappear-

ance of this effect may be the result of changes in the spatial relationships of trp 28, 108 and 63, (fig. 14) which reduce excited state transfer to the latter residue or between trp 63 and the two S–S bridges (fig. 15) resulting in reduced quenching of emission from trp 63. In either case the net effect should be to increase the apparent yield from trp 28. An approximate value of Q_{28} at 77° can be obtained by the method outlined in section 4.2, utilizing yield data for HAL, BAL and GAL (table 2); the value so obtained is 0.01 to 0.02. The yield for the free amino acid at 77° is about 0.025 (fig. 9). Thus, Q_{28} is 40 to 80% of the yield of the free amino acid at 77°. A similar calculation indicates that Q_{28} is only 15–20% of that for the free amino acid at 25°.

3. Since 80% of the fluorescence of the high temperature conformer of GAL can be quenched by I^- (table 4) and only about 20% of the fluorescence from GPAL is so effected in the same state of protein, trp 63 must make a substantial contribution to the emission from GAL at 77° and must also be available for quenching by I^-. As indicated above, the increase in Q_{63} might be due to an absence of quenching by the disulphide bridges in the high temperature conformer. The difference in f_{eff} for HAL and GAL at 77° (table 4) suggests that trp 28 also makes a contribution to the emission from GAL (see above for other evidence for this conclusion) and must also be available to a significant degree for quenching by I^-.

4.6. The N ⇌ U conversion

The low pH conformational change, which we have referred to as the N ⇌ U conversion, occurs with all four α lactalbumins. The pH dependence of the quantum yield for tryptophan fluorescence for BAL and GAL would appear to reflect only this process, while for HAL and GPAL a second pH dependent quenching is superimposed on the increase in Q (fig. 1). Indirect evidence exists for the presence of a small quenching process in BAL and in GAL, but its effect on Q is minimal [5].

The difference spectrum given in curve C, fig. 4 for GPAL appears to correspond primarily to the N ⇌ U conversion, while curve D, having a completely different form reflects the quenching which occurs maximally below pH 3.8. Curve B, fig. 4 for HAL corresponds primarily to the N ⇌ U conversion. The quenching difference spectrum for HAL (pH 3.8–?.5) has an amplitude which lies somewhat above the baseline (data not shown) and falls in the wavelength region 340 to 400 nm.

The quenching observed for HAL and GPAL and inferred for BAL and GAL is likely due to protonation of carboxyls vicinal to tryptophans, comparable to that observed by Lehrer and Fasman [33,34] for hen's egg white lysozyme at acid pH where no conformational change for this protein complicates interpretation of the observations. We have not attempted to identify potentially quenching carboxyl groups in the "lysozyme analogy" model since there is reason to believe that such quenching may actually occur in the U state of the protein whose structure is not known.

It has not been possible to resolve the pH dependence of Q into contributions from the N ⇌ U conversion and the putative carboxyl quenching, but comparison with observations of pH dependence of ϵ_{293} [19] for the N ⇌ U conversion (see below) indicates that the distinctions made above for the difference spectra of HAL and GPAL are reasonable.

Values of $(\Delta Q)_{max}$ (column 2, table 6) were calculated from the pH limits for the N ⇌ U conversion for GAL and BAL, while for HAL and GPAL the limits were taken to be those of the difference spectra of fig. 4. The pH values at midpoints of the Q versus pH and the $\Delta\epsilon_{293}$ versus pH curves [19] are in good agreement for all four proteins. It would appear that the values of $(\Delta Q)_{max}$ given in table 6 are reasonable estimates of the maximum changes occuring in Q for the N ⇌ U conversion.

Comparison of values of $(\Delta Q)_{max}$ and $(\Delta\epsilon_{293})_{max}$ for the four proteins provides some insight into the involvement of specific tryptophan residues in the conformational change. $(\Delta Q)_{max}$ for HAL is at most 25% of the values obtained with BAL and GAL, indicating that trp 28 makes a substantial contribution to the fluorescence change which accompanies the N ⇌ U conversion. $(\Delta\epsilon_{293})_{max}$ observed by Takase et al. [19] for HAL is significantly lower than the values observed for BAL, GAL and GPAL (compare values in column 4, table 6) supporting our conclusion that trp 28 is involved in the N ⇌ U conversion. $(\Delta\epsilon_{293})_{max}$ for GPAL is identical with values found for BAL and GAL, within experimental error, suggesting that the molecular environment of trp 63 undergoes minimal alteration during the N ⇌ U conversion.

Table 6
Comparison of the spectral parameters observed in the N ⇌ U conversion for the four α lactalbumins

Protein	ΔQ_{max} [a]	$(pH)_{1/2}$ [b]	$(\Delta\epsilon_{293})_{max}$	$(pH)_{1/2}$ [f]
(1)	(2)	(3)	(4)	(5)
BAL	0.012	—	2300 [c] 2000 [d]	3.8 [d]
GAL	0.012	3.7	2500 [e]	3.8 [e]
GPAL	0.023	4.5	2200 [c]	4.5 [c]
HAL	0.002–0.003	4.5	1400 [c]	4.5 [c]

a) $(\Delta Q)_{max}$ calculated from Q values in table 1 and fig. 1 for the following values of pH: BAL, 2.5–5.5; GAL, 2.5–4.5; GPAL, 3.8–5.5; HAL, 3.8–5.5.
b) $(pH)_{1/2}$ defined as the pH at which the change in Q is 50% of the maximum value.
c) Data of Takase et al. [19].
d) Data of Kronman et al. [4].
e) Data of Sommers [5].
f) $(pH)_{1/2}$ defined as the pH value at which $\Delta\epsilon$ is 50% of the maximum value.

The latter conclusion might seem to be contradicted by the observations that $(\Delta Q)_{max}$ for GPAL is almost twice that found for GAL and BAL. However, this difference reflects primarily the higher quantum yield of GPAL in both the N and U states; the relative magnitudes of Q for GPAL and GAL or BAL are about the same in the N and U state (table 1, fig. 1). This observation demonstrates that energy transfer from trp 28 and 108 to 63 with subsequent quenching, which we propose occurs in the N state, must occur in the U state as well.

Even though $(\Delta Q)_{max}$ for HAL is relatively small (table 6), the fact that $(\Delta\epsilon_{293})_{max}$ is not equal to zero demonstrates that 28 is not the only tryptophan residue influenced by the N ⇌ U conversion. While we have no experimental evidence to implicate any of the others, a plausible choice is trp 123 located in the "tail" region of the molecule (see above). Warme et al. [23] have pointed out that the conformations T3 and T4 are stabilized by a salt bridge from the ϵ amino group of lys 5 to the terminal carboxyl group of leu 130 and that this salt bridge would likely be broken at either low or high pH. In order to explain the pH dependence of the N ⇌ U conversion we proposed earlier [4] that there were a minimum of three carboxyl groups with abnormally low pK's which became normalized during the acid conformational change. The fact that a process vary similar to the N ⇌ U conversion could be brought about at alkaline pH with normalization of a minimum of two abnormally titrating lysine groups [4]or by acylation of such groups [22], led us to conclude that the abnormal carboxyl and ϵ amino groups were paired in salt bridges. We identified one of the potential carboxyl as the terminal leu [22].

It is of interest to note that reaction of N bromo succinimide with BAL in the N state occurs at trp 123 *prior* to reaction at trp 28 [28]. If the "tail" region of the molecule is in conformation T3 or T4 as we have proposed (section 4.4 above), reaction of trp 123 would require a shift in the conformation to T2 where this residue would be exposed. This shift is comparable to what we have suggested above might occur during the N ⇌ U conversion. This shift in conformation around trp 123 might then facilitate reaction at trp 28 which is buried in the N state. The linkage of conformational changes around trp 123 with these around trp 28, as implied by the above hypothesis, would perhaps also be reflected in their change in conformation during the N ⇌ U conversion. We do not mean to imply that we expect the "tail" region of the α lactalbumin molecule in the U state to be in the T2 conformation with trp 123 exposed. Structural changes in the region of trp 28, however, might be expected to influence the equilibrium conformation for the "tail" region as well.

Some comments are in order concerning observations of exposure of tryptophans in the U state. While solvent perturbation measurements show no net increase in exposed tryptophan groups occur during the N ⇌ U conversion [2,3], two tryptophans form charge transfer complexes with N methyl nicotinamide [32], one more than found in the N state. The difference between these two different kinds of observations appears to lie in the "looser" molecular structure of α lactalbumin in the U state [7,22] which permits greater freedom of motion of aromatic side chains [18,35]. Thus, formation of a charge transfer complex of N methyl nicotinamide with both trp 28 and 123 might occur even if these residues were "buried" in the sense understood by solvent perturbation [30].

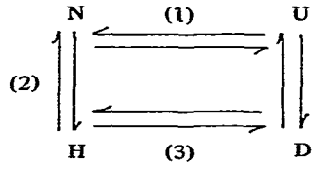
4.7. Folding intermediates of α lactalbumin

We have identified some of the tryptophan residues involved in the N ⇌ U conversion, as well as those implicated in formation of the high temperature conformer. The two processes are sufficiently different to warrent identifying the products as two distinct conformers, U and H:

1. The emission maxima for the H forms of the four proteins lie at longer wavelengths than those observed in the U forms; these in turn exhibit longer wavelength maxima than N forms (compare data of tables 1 and 2). We would conclude from these observations, as well as the iodide quenching data (table 4) that a tryptophan(s) in the H state is exposed to solvent, which does not appear to be the case for the U state. The fact that such emission maxima for the H state are significantly lower than 350 nm, as well as the observation that the extent of quenching is dependent on the wavelength of observation in the emission spectrum (fig. 13), indicates that the H state, however, is not completely unfolded. Kuwajima [7] has summarized observations which support the view that helical regions of the N form persist in the U form (conformer A, in his nomenclature). It will be of interest to determine if this is true in the H form as well, since the latter appears to have a more "open" structure than the U form. Energy transfer to trp 63 with subsequent quenching appears to be largely absent in the H conformer but persists in the U form of the protein.

2. While the fluorescence from trp 28 changes on conversion to H and to U conformers, in the case of the former the increase in yield results indirectly from alteration of the transfer process, while in the latter case emission from the residue appears to be more directly effected.

A plausible pathway for the formation of U and H conformers and subsequent denaturation to form D is



Steps (1) and (2) have been identified by Kuwajima [7] in denaturation of BAL by guanidine-HCl. A pathway of interconversion of U and H forms is ruled out by the absence of a pH dependence of the quantum yield at 60° for GAL and of a *specific* temperature dependent change at pH 2.5. It will be of interest to see if steps (3) and (4) of the above pathway can be accomodated in the kinetic schemes that have been devised to describe the denaturation of α lactalbumin by chemical agents such as guanidine-HCl.

Acknowledgements

We wish to thank Mr. Edward Matyas who wrote the computer programs used to analyse the fluorescence data and Mr. Edward Dixon who constructed the fluorescence spectrometer. Support for this research was provided by NIH Grant #AMI 3021, General Research Grant, NIH 5 Sol RR 5040209 and a Grant from the Research Foundation, State University of New York. Cost of Computer use borne by NIH Grant #RR353 (Biotechnology Branch).

References

[1] U. Brodbeck and K.E. Ebner, J. Biol. Chem. 241 (1966) 762.
[2] M.J. Kronman, L. Cerenkowski and L.G. Holmes, Biochemistry 4 (1965) 518.
[3] M.J. Kronman, W.B. Hoffman, J. Jeroszko and G.W. Sage, Biochim. Biophys. Acta 285 (1972) 124.
[4] M.J. Kronman, J. Jeroszko and G.W. Sage, Biochim. Biophys. Acta 285 (1972) 145.
[5] P.B. Sommers, Ph.D. Dissertation, Upstate Medical Center, State University of New York, Syracuse, NY, U.S.A. (1973).
[6] P.B. Sommers, M.J. Kronman and K. Brew. Biochem. Biophys. Res. Comm. 52 (1973) 98.
[7] K. Kuwajima, J. Mol. Biol. 114 (1977) 241.
[8] D.A. Cowburn, K. Brew and W.B. Gratzer, Biochemistry 11 (1972) 1228.
[9] F.M. Robbins and M.J. Kronman, Biochim. Biophys. Acta 82 (1964) 186.
[10] W.G. Gordon and J. Ziegler, Biochem. Prep. 4 (1955) 16.
[11] K. Brew and P.N. Campbell, Biochem. J. 102 (1967) 258.
[12] J.B.C. Findley and K. Brew, Eur. J. Biochem. 27 (1972) 65.
[13] M.J. Kronman and L.G. Holmes, Photochem. Photobiol. 14 (1971) 113.

[14] R.F. Chen, Anal. Lett. 1 (1967) 35.
[15] J.W. Eastman, Photochem. Photobiol. 9 (1967) 247.
[16] S. Lehrer, Biochemistry 10 (1971) 3254.
[17] A.O. Barel, J.P. Prieels, E. Maes, Y. Looze and J. Leonis, Biochin. Biophys. Acta 257 (1972) 288.
[18] M.J. Kronman, R. Blum and L.G. Holmes, Biochemistry 5 (1966) 1970.
[19] K. Takase, R. Niki and S. Arima, J. Biochem. (Japan) 83 (1978) 371.
[20] M.J. Kronman, R. Andreotti and R. Vitols, Biochemistry 3 (1964) 1152.
[21] W.J. Browne, A.C.T. North, D.C. Phillips, K. Brew, T.C. Vanaman and R.L. Hill, J. Mol. Biol. 42 (1969) 65.
[22] M.J. Kronman, L.G. Holmes and F.M. Robbins, J. Biol. Chem. 246 (1971) 1909.
[23] P.K. Warme, F.A. Momany, S.V. Romball, R.W. Tuttle and H.A. Scheraga, Biochemistry 73 (1974) 768.
[24] J. Eisinger, B. Feuer and A.A. Lamola, Biochemistry 8 (1969) 3908.
[25] R.W. Cowgill, Biochim. Biophys. Acta 140 (1967) 37.
[26] R.F. Steiner and E.P. Kirby, J. Phys. Chem. 73 (1969) 4130.
[27] T.R. Hopkins and R. Lumry, Photochem. Photobiol. 15 (1972) 555.
[28] J.E. Bell, F.J. Castelline, I.P. Travor and R.L. Hill, J. Biol. Chem. 250 (1975) 7579.
[29] T. Imoto, L.N. Johnson, A.C.T. North, P.C. Phillips and J.A. Rupley, The enzymes (Academic Press, New York, 1972) pp. 665–846.
[30] M.J. Kronman and F.M. Robbins, in: The fine structure of proteins and nucleic acids, eds. S.N. Timasheff and G.D. Fasman (Dekker, 1970) pp. 271–407.
[31] T.T. Herskovits, Methods Enzymol. 11 (1967) 748.
[32] F.M. Robbins and L.G. Holmes, J. Biol. Chem. 247 (1972) 3062.
[33] S.S. Lehrer and G.D. Fasman, J. Biol. Chem. 242 (1967) 4544.
[34] S.S. Lehrer and G.D. Fasman, Biochem. Biophys. Res. Comm. 23 (1966) 133.
[35] F.M. Robbins and L.G. Holmes, Biochem. Biophys. Acta 221 (1970) 234.