Randerath, K., and Struck, H. J. (1961), *J. Chromatog. 6*, 365.
Stadtman, T. C. (1960), *J. Bacteriol. 79*, 904.
Toohey, J. I., Perlman, D., and Barker, H. A. (1961), *J. Biol. Chem. 236*, 2119.
Volcani, B. E., Toohey, J. I., and Barker, H. A. (1961), *Arch. Biochem. Biophys. 92*, 381.
Weissbach, H., Ladd, J. N., Volcani, B. E., Smyth, R. D., and Barker, H. A. (1960), *J. Biol. Chem. 235*, 1462.
Wolin, M. J., Wolin, E. A., and Wolfe, R. S. (1963), *Biochem. Biophys. Res. Commun. 12*, 464.

Inter- and Intramolecular Interactions of α-Lactalbumin. III. Spectral Changes at Acid *p*H*

M. J. Kronman,† L. Cerankowski,‡ and L. G. Holmes

ABSTRACT: The denaturation of α-lactalbumin at *p*H values below its isoelectric point is accompanied by blue shifts of the absorption spectrum in the region of 270–300 mμ characteristic of changes in the environment of tryptophan groups. The difference extinction coefficient, ΔE_{293}, is strongly *p*H dependent, but rather insensitive to large changes in ionic strength indicating the probable absence of charge perturbations. In the transition region (*p*H 3–4) ΔE_{293} is also strongly dependent on temperature. The characteristics of this thermal transition indicate that the conformational change associated with the low *p*H denaturation of α-lactalbumin is a complex one involving a series of consecutive or parallel steps. Since the transition at 25° involves the "exposure" of no additional tryptophan groups to the aqueous medium (M. J. Kronman and L. G. Holmes, 1965, *Biochemistry 4*, 526 [this issue; following paper]), it seems probable that the blue shift is the result of alterations of the environment of two or more of the three "buried" groups. It is suggested that this change might come about without unfolding through swelling of the molecule under constraint of the disulfide bridges with subsequent alteration of interactions of "buried" tryptophans with other perturbing groups.

In the first two papers in this series (Kronman and Andreotti, 1964; Kronman *et al.*, 1964), hereafter referred to, respectively, as papers I and II, we demonstrated that below *p*H 4 α-lactalbumin showed a marked propensity toward aggregation (molecular weight of aggregate 3×10^5), reflected as changes with time of the apparent component distribution in the ultracentrifuge and in solubility in ammonium sulfate at *p*H 6.6. In addition, we showed that in the same *p*H range (2–3) association to low molecular weight units could also occur even at protein concentrations where aggregate was absent. In contrast with these observations, aggregation and association were absent or were quite feeble at *p*H values close to but on the alkaline side of the isoelectric point. These observations, together with characteristics of the aggregation process, strongly suggested that the molecular states of monomeric α-lactalbumin were different in these two *p*H regions.

It was suggested (paper II) that this difference was the consequence of a conformational change which made certain groups available for intermolecular interaction, groups which heretofore had been buried within the protein molecule. While at present we cannot demonstrate that such groups are liberated during this denaturationlike process, we have sufficient information to support our hypothesis that a conformational change has occurred. For example, at *p*H 2 at protein concentrations where aggregation and association are absent, sedimentation velocity measurements reveal an increase in the frictional ratio of the monomeric α-lactalbumin molecule consistent with an increase in its hydrodynamic volume (paper II). Further evidence for such a conformational change has been obtained from rotatory dispersion measurements (M. J. Kronman and R. Blum, unpublished experiments) and changes in the ultraviolet fluorescence of tryptophan residues (M. J. Kronman, manuscript in preparation). These changes, as well as the spectral properties to be reported here, are a reflection of shifts in the balance of intramolecular interactions that stabilize the protein molecule in its native state. In this series of papers we shall attempt to describe the relationship between

* From the Pioneering Research Division, U.S. Army Natick Laboratories, Natick, Mass. (M. J. K. and L. G. H.) and the Eastern Regional Research Laboratory, U.S. Department of Agriculture, Philadelphia, Pa. (M. J. K. and L. C.). *Received October 26, 1964.* Presented in part before the Division of Colloid Chemistry, at the National Meeting of the American Chemical Society, Los Angeles, Calif., April 1963.

† Present address: Pioneering Research Division, U.S. Army Natick Laboratories, Natick, Mass.

‡ Present address: Department of Chemistry, Princeton University, Princeton, N.J.

*inter*molecular interactions (reflected as changes in the state of aggregation of the protein) and the alteration of these stabilizing *intra*molecular interactions.

Ultraviolet spectra provide a sensitive means of detecting conformational changes in proteins in the vicinity of aromatic amino acid residues. α-Lactalbumin below its isoelectric point exhibits temperature and *p*H-dependent spectral shifts both in the wavelength region clearly associated with tyrosine and tryptophan absorption (>250 mμ) and in the low-wavelength region (<250 mμ) where spectral changes may be identified with a variety of protein chromophores. The features of these spectral changes in α-lactalbumin, as well as their relationship to alteration of tryptophan environment, are presented here.

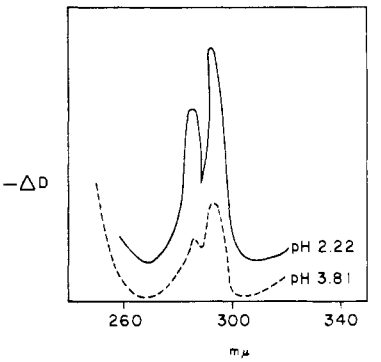

FIGURE 1: Difference spectra of α-lactalbumin. Protein concentration *ca.* 0.09 g/100 ml; temperature, 25°; 0.15 M KCl.

Experimental

Materials. α-Lactalbumin prepared by the methods of Aschaffenburg and Drewry (1957), Gordon and Semmett (1953), and Robbins and Kronman (1964) was employed. The characteristics of these three preparations, designated, respectively, 3088, 3665, and R49, have been described in papers I and II. Identical spectral properties were found with all preparations. Glass-distilled water and reagent grade chemicals were used in all experiments.

Preparation of Solutions. α-Lactalbumin stock solutions were prepared by dissolving the appropriate amount of protein slurry in a solution of the desired salt concentration. Dialysis to remove ammonium sulfate was then carried out at 1–2° for 24 hours using a minimum of three changes of 150-fold excesses of solvent. This stock solution was diluted and adjusted to the *p*H required, as indicated.

Preparation of acidified solutions of α-lactalbumin for differential spectral measurements is quite difficult since near irreversible turbidity is readily produced on passing through the region of low solubility (*p*H 4–5.5) unless special precautions are observed. Attempts to prepare optically clear solutions of α-lactalbumin at *p*H values above 5 by addition of base to an acidified solution were never successful. The following procedure, carried out in the sequence indicated, generally yielded satisfactory results: (a) 2.00 ml of the dialyzed protein stock solution was added to a 25-ml volumetric flask. (b) About 15 ml of the diluting electrolyte was added. (c) The required increment of 1 N HCl was added rapidly with efficient mixing of the flask contents by swirling. (d) The solution was brought to the mark with the electrolyte solvent. For *p*H values between 3 and 4 it was necessary to carry out all of the above manipulations in the cold room (1–2°) to avoid turbidity. Reference solutions which contained no acid were prepared from the same stock as the series of acid solutions, using identical volumetric glassware. The *p*H values of reference solutions ranged from 5.6 to 6.0. Protein concentrations were determined by ultraviolet absorption of the diluted stock solutions using a value of $E^{1\%}_{1cm}$ of 20.1 (paper I). Measurements of *p*H with the Beckman Model G meter were made soon after preparation of protein solutions and frequently after completion of a series of spectral measurements (*ca.* 8 hours later).

Difference Spectral Measurements. A Beckman DU spectrophotometer equipped with thermospacers and photomultiplier tube was employed. Circulation of water from a refrigerated constant-temperature bath permitted measurements to be made over the temperature range 2–35°. The temperature of the cell contents was taken as the average of the temperatures of the constant-temperature fluid entering and leaving the thermospacers. The accuracy of the temperatures reported is ±1° at 0° and ±0.25° at 25°.

For measurements initiated at 25° solutions were equilibrated in the spectrophotometer for 15 minutes prior to measurement. Measurements for solutions prepared in the cold room (*p*H 3–4) were initiated at 1–2° in the precooled spectrophotometer. Cells containing these solutions were transferred from the cold room to the cell compartment of the instrument in a covered Dewar flask. The instrument was purged with clean dry nitrogen or air to eliminate moisture during all operations.

Measurements in the wavelength region above 250 mμ were usually made on protein concentrations below 0.09 g/100 ml to eliminate effects of stray light and of possible association (paper I). Measurements carried out below 250 mμ employed solutions of concentration 0.009 g/100 ml or less. The data were calculated as $\Delta E^{1\%}_{1cm}$.

In addition to visual checks on turbidity, each solution was examined frequently during the course of the measurements at wavelengths above 320 mμ where absorption is absent. Observation of ΔD values greater than ±0.003 in this wavelength region were grounds for rejection of that set of data. These checks were particularly important at *p*H values above 3.5 when temperatures near ambient were approached.

Results

Difference Spectra of α-Lactalbumin. Comparison of spectra of α-lactalbumin at low *p*H with those obtained

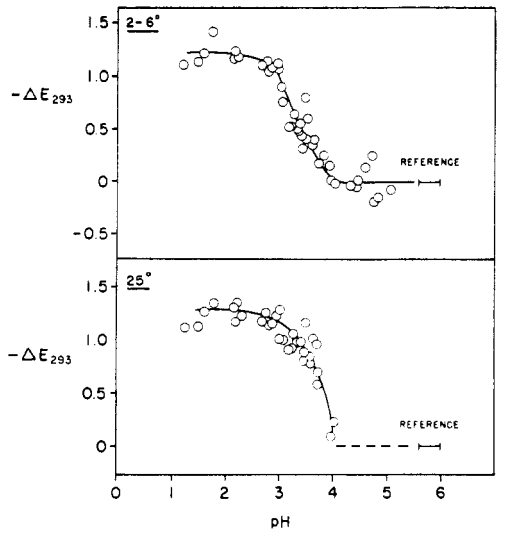

FIGURE 2: *p*H dependence of ΔE_{293} at 2–6° and 25°. Other conditions as in Figure 1.

near *p*H 6 reveals changes in two wavelength regions: near 230 mμ and in the 270- to 300-mμ region. The latter obtained as difference spectra are shown in Figure 1. The lower wavelength changes will be considered in a subsequent section. A *p*H range of 5.6–6 was chosen as a reference point since from the viewpoint of the aggregation-solubility behavior, α-lactalbumin is "most native" in this region (paper II). These blue-shifted difference spectra show major peaks at 285–286 and 292–293 mμ. The amplitude of these peaks was found to depend upon both temperature and *p*H (Figure 1). In all cases examined the spectral changes had occurred within five minutes after *p*H adjustment.

*p*H *Dependence in the 270- to 300-mμ Region.* Since α-lactalbumin contains five tyrosines and five tryptophans out of a total of 126 residues (Gordon and Ziegler, 1955), the spectrum in the wavelength region 270–300 mμ will be largely due to tryptophan absorption. The 293-mμ peak of the difference spectrum illustrated in Figure 1 is thus a reflection of alterations in the environment of tryptophan residues in the protein molecule as the protein is brought from *p*H 6 (reference *p*H) to acid *p*H.

In the *p*H region 3–4, ΔE_{293} is strongly *p*H dependent both at low temperature (2–6°) and near room temperature (25°) (Figure 2). In the isoelectric region (*p*H 4–5) at low temperature ΔE_{293} is essentially zero. The precision is low in this *p*H region since the low solubility of the protein requires that measurements be made on solutions of rather low concentration (<0.05 g/100 ml). For the same reason measurements at higher temperatures in this *p*H region cannot be made at all.

The *p*H dependence of the molecular transformation is quite sharp. As Figure 2 illustrates (at low temperature) about 75% of the change in differential extinction coefficient occurs between *p*H 3 and 4. Below *p*H 3 there is a further small change but ΔE_{293} levels off and is relatively constant to *p*H values below 2. At 25° a similar sharp transition is observed in the *p*H region 3–4, with ΔE_{293} leveling off at lower *p*H to values slightly higher than those encountered at low temperature. In the transition region ΔE_{293} values are higher at a given *p*H than those observed at lower temperature. As a measure of this it may be seen that at 25°, 50% of the change in ΔE_{293} occurs by *p*H 3.7–3.8 while at 2–6° the *p*H required to effect a similar change is about *p*H 3.3.

Changes in ΔE_{286} essentially paralleled those for the 293-mμ peak, as one might anticipate from the dominant character of tryptophan in the spectrum of α-lactalbumin. Below *p*H 3.25 there was some indication of a nonparallel change, i.e., ΔE_{286} decreased somewhat slower than ΔE_{293} with increase in *p*H. Likewise, there seemed to be some persistence of the 286-mμ peak (ΔE_{286} = 0.1–0.2) at low temperature to *p*H values near 5. Unfortunately, as mentioned before, in this *p*H range ΔD is quite small and the precision of the measurement is rather poor. The involvement of tyrosine residues in the transition must therefore remain, at this point, conjectural.

Reversal of Molecular Transformation. The molecular transformation associated with the spectral changes appears to be completely reversible as was the case for the time-dependent aggregation described in paper II. This was demonstrated in the following way: Protein solutions of appropriate concentration were allowed to stand at *p*H values between 1.5 and 3 for 2–3 hours. Sufficient 1 N KOH was then added to bring the solution to *p*H 6. Unavoidably, however, as the solution is titrated through the isoelectric region toward alkaline *p*H values, a small amount of turbidity is produced which makes direct spectral comparison with α-lactalbumin (untreated with acid) impossible (see also Experimental). To circumvent this difficulty the following procedure was employed: The haze was removed by centrifugation at 144,000 *g* for 15 minutes; the change in concentration resulting from this treatment was less than 2%. An aliquot of this solution was then taken to *p*H 2 in the usual way and compared with a similar dilution of the *p*H 6 centrifuged solution. The difference spectrum obtained in this type of experiment was identical with those obtained with protein that had not been acid exposed prior to measurement. The reversibility of the molecular transformation is also seen on comparison of spectra obtained with α-lactalbumin prepared by different procedures (see Experimental). Samples 3088 and 3665 were obtained by the method of Aschaffenberg and Drewry (1957) which involves in an early step precipitation of the protein at *p*H 2 with 2.1 M sodium sulfate. Sample R49 was prepared by a procedure which avoids acid treatment (Robbins and Kronman, 1964). Differential spectra obtained with all three preparations were identical proving that acid treatment does not produce irreversible changes. Unfortunately these methods used to test reversibility reveal nothing about the rate of reversal.

Temperature Dependence of the Spectral Transition. The differences in the *p*H dependencies of ΔE_{293} at 2–6° and at 25° (Figure 2) are reflections of a significant

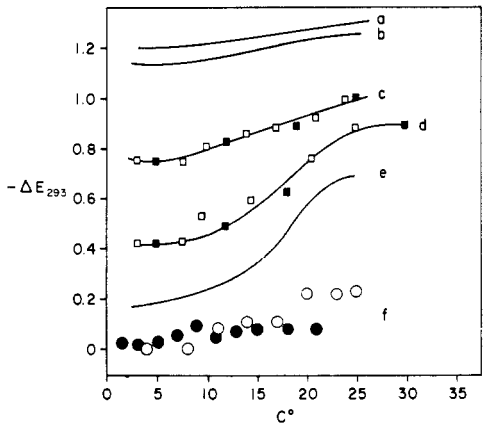

FIGURE 3: Temperature dependence of ΔE_{293} at various *p*H. Solid and open squares are data obtained on heating and cooling cycles. The solid lines were obtained from smoothed data obtained in heating and cooling cycles. (*a*) *p*H 2.17, (*b*) *p*H 2.78, (*c*) *p*H 3.07, (*d*) *p*H 3.49, (*e*) *p*H 3.78, (*f*) *p*H 4.03; solid circles, protein concentration, 0.144 g/100 ml; open circles, 0.0905 g/100 ml. In *a–e*, protein concentration *ca.* 0.09 g/100 ml. Other conditions as in Figure 1.

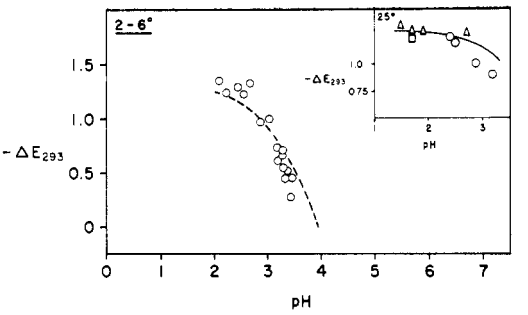

FIGURE 4: Ionic strength dependence of ΔE_{293}, main graph 2–6°, insert 25°. Electrolyte KCl. ○, 0.05 M; □, 0.50 M; △, 0.75 M. Solid lines represent smoothed data for 0.15 M KCl taken from Figure 2. Protein concentration *ca.* 0.09 g/100 ml.

dependence of the transition on temperature in the *p*H region 3–4. Representative curves illustrating this are shown in Figure 3. While ΔE_{293} is somewhat temperature dependent below *p*H 3 (curves *a* and *b*), a marked dependence is seen at *p*H values above this value (curves *c*, *d*, *e*). The reversibility of the thermal transition is seen on comparison of the filled and unfilled points plotted on curves *c* and *d* which represent values obtained on successive heating and cooling cycles. This reversal could always be accomplished except above *p*H 3.5, where exposure of the solutions to temperatures approaching 25° generally resulted in the appearance of turbidity which precluded further measurements. Implicit in this type of measurement is the assumption that the temperature dependence of the difference spectrum is a reflection of changes occurring at acid rather than at the reference *p*H (5.6–6). This was verified by comparison of the absolute spectra at *p*H 5.6–6 at 4° and 25°. The change in $E^{1\%}_{1cm}$ (obtained in replicate measurements) at 293 mμ was -0.01 ± 0.02. The anticipated change in extinction coefficient resulting from volume changes is about +0.04. These latter observations have been recently confirmed by Mr. R. Biltonen in Dr. R. Lumry's laboratory using a Cary Model 15 recording spectrophotometer. Thus, the spectral changes observed in acid difference spectra with changing temperature must be the result of changes in spectra of acidified protein solutions.

Of particular interest in the *p*H 3–4 thermal transition is the fact that ΔE_{293} values at different *p*H values do not approach common values at low temperature. As the curves of Figure 3 indicate, they level off below 6° to ΔE values which are successively higher as the *p*H is increased. A similar situation may be true as the temperature is increased, i.e., ΔE_{293} appears to level off at successively higher values with increasing *p*H. Unfortunately, no rigorous test of the latter is possible owing to the development of turbidity at the higher temperatures.

The filled and unfilled circles in curve *f* represent two sets of measurements carried out at *p*H 4.03 at protein concentrations of 0.144 and 0.0905 g/100 ml, respectively. Aside from the deviation observed above 20° the agreement is good. This deviation above 20° is not surprising since the measured optical densities are small (~0.02) and errors owing to turbidity are apt to be large under these conditions. Similar measurements made at other *p*H values indicated that, within experimental error, Beer's law is obeyed.

It seems unlikely, therefore, that the association-aggregation phenomena per se (papers I and II) have a significant effect on the difference spectra at the protein concentrations employed in this study. Further evidence to support this conclusion is seen in the absence of a time dependence of the spectral change, in contrast with the aggregation process which is clearly time dependent (paper II). Furthermore, measurements made by the short-column equilibrium sedimentation procedure at protein concentrations somewhat higher than those employed in this study (*ca.* 0.2 g/100 ml) yielded molecular weights at *p*H 3, 2, and 6 approaching that of the monomer, i.e., 75–95 mole % of α-lactalbumin was found to be monomer (paper I). These values were calculated from the weight-average molecular weights assuming a monomer-dimer equilibrium and a molecular weight of 17×10^3 for the monomer. The latter value was obtained at a protein concentration of 0.2 g/100 ml at *p*H 8.55 (paper I). Thus, at the still lower concentrations employed in this study (*ca.* 0.1 g/100 ml), association should be essentially absent. The spectral changes must therefore be caused by the conformational change.

Effect of Ionic Strength. Alteration of ionic strength has a minimal effect on the difference spectra observed in acid solution. Shown in Figure 4 are ΔE_{293} data obtained as a function of *p*H at low temperature and at 25°

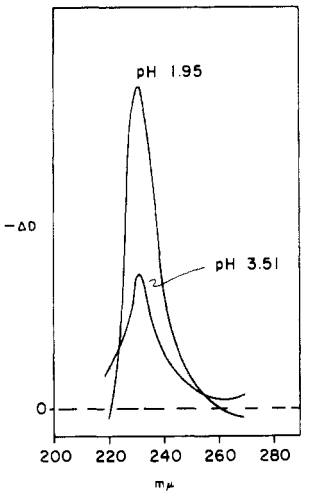

FIGURE 5: Difference spectra of α-lactalbumin in the low-ultraviolet region. Protein concentration *ca.* 0.009 g/100 ml; temperature 3°; 0.15 M KCl.

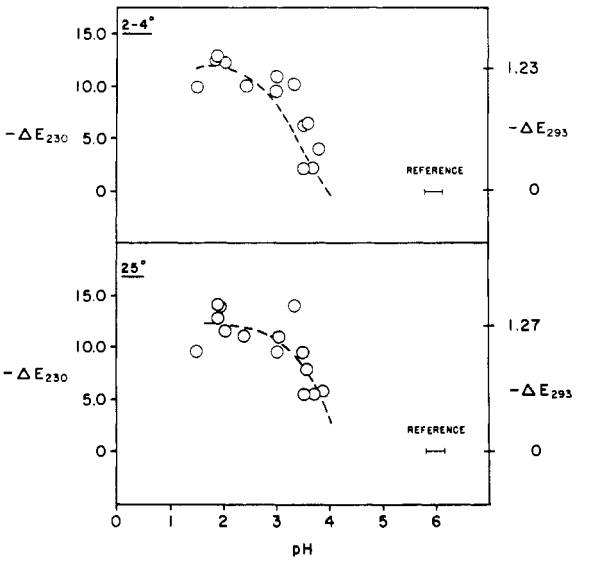

FIGURE 6: *p*H dependence of ΔE_{230} at 2–4° and 25°. Other conditions as in Figure 5. Dotted line represents smoothed ΔE_{293} data taken from Figure 2. See text for explanation of fitting procedure.

(insert) for a 15-fold change in Γ/2. Also included for comparison is the smoothed data of Figure 2 for 0.15 M KCl (solid lines). As the data of the main graph of Figure 4 illustrate, a decrease in ionic strength serves chiefly to shift the transition curve to slightly lower *p*H. In the *p*H region below 3 a decrease in ionic strength from 0.15 (solid curve) to 0.05 yields a very small increase in ΔE_{293}. Similar results were obtained at 25° (Figure 4, insert) where a 15-fold increase in ionic strength has little or no effect on the value of ΔE_{293} at low *p*H.

Spectral Changes in the 230-mμ Region. Acid difference spectra below 250 mμ reveal a maximum around 230 mμ (Figure 5). The *p*H dependence of the amplitude of the 293-mμ peak roughly paralleled that at 230 mμ (Figure 6). The circles in Figure 6 represent the 230-mμ data while the dotted curves describe the smoothed 293-mμ data (Figure 2). The ordinates were adjusted to bring the zero points and the plateau regions for the two sets of data into registration. The significance of the deviation of the 230-mμ data from the 293-mμ curves is difficult to assess since turbidity errors incurred above *p*H 3 are apt to be quite significant at low wavelengths. Nonetheless, it is reasonable to associate the two spectral changes with the same molecular process.

Abolition of the Difference Spectrum. Exposure of α-lactalbumin to 8 M urea destroys the characteristic acid difference spectrum. Shown in Figure 7 is a comparison of the spectrum for the native protein (curve *a*) with that for protein denaturated in 8 M urea (curve *b*). In the latter case both the reference solution (*p*H 6) and the acid solution contained 8 M urea. The spectrum for urea-denatured protein (curve *b*) is a featureless curve comparable to that obtained by comparison of a protein-free 8 M urea solution at *p*H 3.4 with one at *p*H 6 (curve *c*). Rotatory dispersion measurements in 8 M urea yield b_0 values of about −50° indicating disruption of the organized molecular structure (M. J. Kronman and R. Blum, unpublished experiments). Under these conditions the denaturation appears to be instantaneous. Thus, the generation of difference spectra such as those shown in Figure 1 must require the existence of the characteristic conformation of the native protein. Measurement of the difference spectrum of native α-lactalbumin versus α-lactalbumin denatured in 8 M urea yielded a value of $\Delta\epsilon_{293}$ of −3200.

Discussion

Changes in Conformation of α-Lactalbumin. The spectral changes observed in this study (Figures 1 and 5) are clearly the result of conformational changes occurring in α-lactalbumin. The changes in amplitude of the difference peaks occur largely in the same *p*H region, 3–4 (Figures 2, 4, and 6), as do changes in tryptophan fluorescence (M. J. Kronman, manuscript in preparation), rotatory dispersion parameters (M. J. Kronman and R. Blum, unpublished experiments), and in the titration curve electrostatic factor, *w* (F. M. Robbins, R. E. Andreotti, and M. J. Kronman, unpublished experiments). Likewise, it is within this *p*H region that we observed increasing susceptibility to aggregation (paper II).

The interpretation of spectral changes occurring in the 230-mμ region (Figure 7) is particularly difficult owing to the contributions of a number of chromophores, including tyrosine and tryptophan, to the absorption in this wavelength region (Wetlaufer, 1962). Glazer and Smith (1961) have attributed the 230-mμ difference peak to changes in peptide backbone conformation, although they also point out that it may have other origins as well. While rotatory dispersion changes,

possibly indicative of peptide backbone conformational changes, do occur with α-lactalbumin on acidification (M. J. Kronman and R. Blum, unpublished experiments), the total decrease in b_0 of 50–60° appears to be too small to account for the large change in absorption ($\Delta\epsilon_{230} \sim -20{,}000$). By contrast, the change in $\Delta\epsilon_{230}$/residue observed by Glazer and Smith (1961) for polyglutamic acid was about 85 for a change in b_0 of about 600°. Thus, for α-lactalbumin at least, it seems more reasonable to associate the 230-mμ changes with those observed at 293 mμ which are clearly identified with alterations of tryptophan environment.

pH and Ionic Strength Dependence of the Conformational Change. The *p*H dependence of the spectral transition does not correspond to the titration curve of carboxyl groups, since by *p*H 4, where the shift begins, almost 50% of the carboxyl groups are protonated (F. M. Robbins, R. E. Andreotti, and M. J. Kronman, unpublished experiments). The location of the transition in this *p*H region, however, does imply a participation of carboxyl groups in maintaining the native conformation, although the nature of their role is not at all evident.

The absence of any significant dependence of ΔE_{293} on ionic strength at low *p*H (Figure 4) makes it unlikely that the conformational change depends to a significant extent upon intramolecular charge repulsions as is the case, for example, with the low-*p*H conformational change of bovine serum albumin (Kronman and Foster, 1957). The shift of the transition curve to lower *p*H with decrease in ionic strength (Figure 4) is consistent with the anticipated shift of the titration curve of carboxyl groups.

Temperature Dependence of the Conformational Change. If we assume the absence of abnormally titrating groups such as histidine or lysine, the location of the spectral transition in the *p*H region 3–4 (Figure 2) implicates the titration of carboxyl groups in the conformational change. If protonation of such groups were the only process involved in the spectral transition we would anticipate a zero or small positive enthalpy (*ca.* +2 kcal) (Tanford, 1962). The enthalpy calculated from apparent *pK* values obtained from the curves of Figure 2 are of the order of −10 kcal. This negative enthalpy cannot be owing solely to carboxyl protonation and must correspond to other molecular processes.

The vaue of −10 kcal given is actually an apparent enthalpy which is the resultant of values corresponding to more than one molecular process. This can be seen by a comparison of the ΔE_{293} versus temperature curves for different *p*H values (Figure 3). The fact that these curves do not come to common values at low temperature, but level off at successively higher temperatures as the *p*H is decreased, cannot be reconciled with a one-step process and indicates that no simple thermodynamic analysis is possible.

While other possibilities cannot be excluded, the simplest explanation for the complex character of the thermal transition is that it involves simultaneous or closely consecutive alterations of the molecular conformation in regions adjacent to different tryptophan residues. As we shall see subsequently, it probably involves at most three of the five groups in α-lactalbumin.

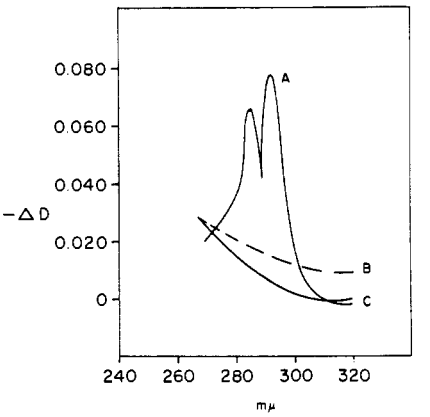

FIGURE 7: Effect of urea on acid difference spectra at 25°. (A) At *p*H 3.2, urea free; (B) *p*H 3.4, 8 M urea; (C) *p*H 3.4, 8 M urea. Protein absent from both sample and reference. Other conditions as in Figure 1.

Molecular Origin of the Low-pH Spectral Shift. While the spectral shifts observed at acid *p*H appear to be the consequence of a conformational change, the manner in which this change alters the environment of tryptophan groups is not immediately obvious because of the numerous ways in which such shifts can arise (Yanari and Bovey, 1960). The most commonly invoked explanation of denaturation blue shifts (see, for example, Figure 1), i.e., an unfolding of the protein molecule to expose the chromophore to solvent, has been ruled out by the recent solvent perturbation measurements of Kronman and Holmes (1965). These data showed that at 25° three of the five tryptophan groups in α-lactalbumin are completely buried. With none of the perturbants, including heavy water, were we able to detect an increased exposure of groups in going from *p*H 6 to low *p*H. Thus the blue shift observed at 25° cannot be the consequence of a transfer of tryptophans from regions of high polarizability within the molecule to contact with the aqueous medium.

Similarly at low temperature, while large perturbants such as sucrose and glycerol indicated a more restricted access of the two exposed groups at low temperature than at 25°, they remained exposed to heavy water both at *p*H 6 and below the transition region (*p*H 1.8–3). Since the "burying" of groups (with respect to large perturbants) with decrease in temperature is accompanied by a very small spectral shift at low *p*H (curve *a*, Figure 3) and none at *p*H 6 (see Results: *Temperature Dependence of the Spectral Transition*), it seems likely that the large spectral shifts observed on acid denaturation of α-lactalbumin arise from changes in the environment of the three "*buried*" groups.

The increase in frictional ratio (paper II) and the decrease in *w* obtained from titration of carboxyl groups (F. M. Robbins, R. E. Andreotti, and M. J. Kron-

man, unpublished experiments) indicate that below the transition region the α-lactalbumin molecule exists in a more swollen state than at *p*H 6. This swelling should lead to greater freedom of motion of side chains within the molecule. The following represent possible explanations of the observed blue shift consistent with increased freedom of rotation in the denatured state: (a) alterations of perturbation of buried tryptophans by the field of charged functional groups, (b) breaking of hydrogen bonds from the indole nitrogens to suitable acceptors, (c) local changes in polarizability or in dipolar interactions with tryptophan groups.

The first mechanism (electrostatic perturbation) appears to underlie the small blue shift observed for lysozyme since solvent perturbation measurements indicate about 75% exposure both at acid *p*H and near neutrality (Laskowski *et al.*, 1962). It seems less plausible for α-lactalbumin. The blue shifts observed for α-lactalbumin at acid *p*H and 25° differ in several ways from those attributed to charge perturbation in lysozyme. The differential extinction coefficient for α-lactalbumin ($\Delta\epsilon_{293} = -403$) is markedly larger than that observed for lysozyme ($\Delta\epsilon_{293} = -80$) (Yanari and Bovey, 1960), and is relatively insensitive to a 15-fold change in ionic strength (Figure 4). For lysozyme a comparable increase in ionic strength produces a nearly 2-fold change in $\Delta\epsilon_{293}$ (Donovan *et al.*, 1961). The absence of an ionic strength dependence for α-lactalbumin, while not conclusively eliminating charge perturbations as a source of the spectral shift, demonstrates that if they are operative they are not propagated through the medium where screening by salt might be anticipated. However, it does not rule out the possibility of perturbation of the three buried groups by electrostatic effects propagated through nonpolar regions of the molecule, where the low dielectric constant of such regions would tend to enhance such effects as compared to similar perturbations propagated through the aqueous medium. The fact that the difference spectrum is abolished in the presence of 8 M urea (Figure 7) indicates that charge effects, if present, are not the consequence of a favorable distribution of groups along the peptide chain but require the specific three-dimensional geometry determined by the native structure. Finally, the presence of a temperature-dependent spectral transition in the carboxyl titration region (Figures 2 and 3) is inconsistent with a charge perturbation per se since the enthalpy of ionization of carboxyl groups is close to zero (Tanford, 1962). The temperature dependence could be accounted for, however, by the occurrence of a temperature-dependent conformational change which might provide the appropriate geometry for coulombic perturbations.

The breaking of hydrogen bonds from indole to an acceptor such as a peptide oxygen atom should lead to a blue shift. Yanari and Bovey (1960) concluded from a study of the ultraviolet spectra of indole in ethanol and isooctane that such bonds form and yield a red-shifted spectrum. Lumry and co-workers (communication from Dr. R. Lumry) have likewise shown by infrared measurements that indole is capable of forming such hydrogen bonds. While such bonds may have only marginal stability in aqueous medium, the low polarity of the interior of a protein molecule would favor their formation (Franzen and Stephan, 1963).

The low quantum yield of tryptophan in α-lactalbumin (M. J. Kronman, manuscript in preparation) suggests that the three buried groups are in intimate contact with quenching groups (perhaps peptide oxygen atoms) and that the low-*p*H denaturation involves a decrease in such contacts. Since the dipole moment of the indole moiety is increased in the excited state (R. Lumry, personal communication), we anticipate that any decrease in contact between tryptophan residues and polar groups resulting from increased free rotation will result in a reduction of dipole-dipole interactions and hence yield a blue shift of the absorption spectrum relative to the native protein. Whether such local dipolar interactions or local changes in polarizability can produce changes of the magnitude reported here cannot be answered at present. This same uncertainty makes it impossible to distinguish between the various mechanisms suggested in explanation of the observed spectral shifts.

Magnitude of Shifts on Exposure of Groups. While quantitative prediction of spectral shifts seems impossible at present, it is nonetheless instructive to compare the magnitude of the observed values with those obtained in cases where "exposure" of tryptophan groups can be clearly established. Such a comparison can be made for acid- and urea-denatured α-lactalbumin. In the latter case we found that treatment with 8 M urea resulted in a change in "exposure" of groups which can be interpreted as complete "exposure" of two, or 70% "exposure" of three additional groups (Kronman and Holmes, 1965). Calculation of $\Delta\epsilon_{293}$/group "exposed" for urea denaturation yielded values of −1900 to −2800, depending upon whether we assumed that two or three additional groups were completely "exposed." These values were obtained from the observed value of −3200 after correction for the red shift experienced by tryptophan on transfer from water to 8 M urea. The spectral data of Bigelow and Geschwindt (1960) for *N*-acetyltryptophan ethyl ester in water and 7.5 M urea was used for the latter correction. Similarly, computations made for the acid denaturation of aldolase using the spectral and solvent perturbation data of Donovan (1964) yielded a value of $\Delta\epsilon_{293}$/group of about −2000 (exposure of nine groups).

In contrast with these values of −2000/ to −3000/ group observed on exposure of tryptophans, acid denaturation of α-lactalbumin yielded a value of about −670/group (based on three groups). While these computations are of necessity rather crude ones, they serve to illustrate that the spectral changes occurring on acid denaturation of α-lactalbumin are considerably smaller than those which *clearly* involve an unfolding of the molecule with concomitant exposure of chromophores. Whether the magnitude of spectral shifts might be generally useful in distinguishing unfolding from less drastic processes is something that will require investigation with other tryptophan-containing proteins.

The observations made in this study and in the parallel solvent perturbation investigation (Kronman and Holmes, 1965) are a compelling illustration of the dangers inherent in attributing spectral shifts to "exposure" of chromophores in the absence of corroborative evidence such as solvent perturbation. Used in a complimentary way, difference spectroscopy and solvent perturbation will permit the detection of conformational changes and the verification if such spectral changes are due to "exposure" of groups. Only in this way is it possible to distinguish those conformational changes which involve unfolding of the molecule from those which involve alterations which, for lack of further information, we term "internal changes." This distinction is apt to be quite significant since, as we have shown in this study, relatively large spectral shifts need not be the result of unfolding, even when the process bears all the earmarks of denaturation.

Acknowledgments

We are pleased to acknowledge a number of stimulating conversations with Dr. Rufus Lumry and Dr. Theodore Herskovits concerning our results. We also wish to thank Mr. R. Biltonen for carrying out some confirmatory measurements.

References

Aschaffenburg, R., and Drewry, J. (1957), *Biochem. J. 65*, 273.

Bigelow, C. C., and Geschwindt, I. I. (1960), *Compt. Rend. Trav. Lab. Carlsberg 31*, 283.

Donovan, J. W. (1964), *Biochemistry 3*, 67.

Donovan, J. W., Laskowski, M., Jr., and Scheraga, H. A. (1961), *J. Am. Chem. Soc. 83*, 2686.

Franzen, J. S., and Stephan, R. E. (1963), *Biochemistry 2*, 1321.

Glazer, A. N., and Smith, F. L. (1961), *J. Biol. Chem. 236*, 2942.

Gordon, W. G., and Semmett, W. F. (1953), *J. Am. Chem. Soc. 75*, 328.

Gordon, W. G., and Ziegler, J. (1955), *Arch. Biochem. Biophys. 57*, 80.

Kronman, M. J., and Andreotti, R. E. (1964), *Biochemistry 3*, 1145.

Kronman, M. J., Andreotti, R. E., and Vitols, R. (1964), *Biochemistry 3*, 1152.

Kronman, M. J., and Foster, J., F. (1957), *Arch. Biochem. Biophys. 72*, 205.

Kronman, M. J., and Holmes, L. G. (1965), *Biochemistry 4*, 526 (this issue; following paper).

Laskowski, M., Jr., Herskovits, T. T., and Williams, E. J. (1962), Abstracts of Papers, 142nd Meeting, American Chemical Society, Atlantic City, N.J., September 1962.

Robbins, F., and Kronman, M. J. (1964), *Biochim. Biophys. Acta 82*, 186.

Tanford, C. (1962), *Advan. Protein Chem. 17*, 69.

Wetlaufer, D. B. (1962), *Advan. Protein Chem. 17*, 304.

Yanari, S., and Bovey, F. A. (1960), *J. Biol. Chem. 235*, 2818.