Inter- and Intramolecular Interactions of \( \alpha \)-Lactalbumin

X. EFFECT OF ACYLATION OF TYROSYL AND LYSYL SIDE CHAINS ON MOLECULAR CONFORMATIONS

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SUMMARY

Of the 4 tyrosyl residues of bovine \( \alpha \)-lactalbumin, 2 were found to be quite reactive with \( N \)-acetylimidazole; the third group is less so, and the fourth is only weakly reactive. Acylation of the two most reactive groups results in a reversible increase in the quantum yield of a tryptophan residue or residues which emit maximally at 350 \( \mu \)m. At relatively high concentrations of reagent, in addition to acylation of tyrosyl residues, six amino groups react giving rise to quenching of the fluorescence of tryptophan emitting maximally below 320 \( \mu \)m and enhancement of fluorescence from groups which emit maximally at 350 \( \mu \)m. Thus, acylation of the two tyrosyl groups perturbs tryptophan residues which are freely in contact with the medium, whereas acylation of amino groups, by contrast, appears to involve tryptophan residues which are not freely in contact with the solvent.

Changes in circular dichroism were observed on acylation of bovine \( \alpha \)-lactalbumin in the 250 to 300 \( \mu \)m side chain band system, in the 206 \( \mu \)m band, and in the 212 to 230 \( \mu \)m region. These circular dichroism changes, which depend in a complex way on the nature of the groups acylated, appear to be due primarily to alteration of the environment of the side chains in the protein.

Changes in fluorescence and circular dichroism properties observed on acylation of amino groups were shown to be similar to those observed on acid denaturation. It is likely that those processes, as well as alkaline denaturation, bring about essentially the same local conformational change which gives rise to increased freedom of rotation of tyrosyl and tryptophyl side chains. The pH dependence of the conformational transitions, as well as the chemical reactivity of side chains, suggests that breaking of charge pairs (amino and carboxylic acid groups) may initiate the structural change.

Inspection of a recently proposed model for bovine \( \alpha \)-lactalbumin indicates the proximity of a number of lysyl, glutamyl, and aspartyl side chains, in accord with the above hypothesis. The model further indicates that Tyr-36 and Trp-118, and Tyr-103 and Trp-104 are sufficiently close, respectively, to explain the changes in fluorescence on acylation of tryrosine groups. The environments of Trp-60 and perhaps Trp-104, located in a crevice-like region of the molecule, are such that they may be the low wave length emitting groups normalized on acylation of amino groups.

In previous papers (1-9) we have been concerned with characterization of bovine \( \alpha \)-lactalbumin, particularly with regard to the various structural changes that it undergoes with alteration of solution parameters. The discovery of the correspondence of this protein and Component B of lactose synthetase (10, 11) and the elucidation of its participation in the synthesis of lactose as a substrate specifier for uridine diphosphate galactose: \( \alpha \)-glucose 1-galactosyltransferase (EC 2.4.1.22) (12) (Component A of lactose synthetase) make studies of this protein of great importance in ultimately relating its unique control function and its structural properties in solution.

Another very important development which underlies the significance of structural studies of \( \alpha \)-lactalbumin is the observation of the homology of the amino acid sequence of the bovine protein with that of egg white lysozyme (13-16). The implication of these observations is that the three-dimensional structure of the two proteins should show great similarity. Model-building experiments (17) have shown that it is possible to fold the \( \alpha \)-lactalbumin amino acid sequence to the known conformation of the lysozyme backbone (18). A comparison of the circular dichroic spectra has indicated that no gross differences in backbone conformation exist in solution for these two proteins (19).

Studies of the acylation of \( \alpha \)-lactalbumin were originally undertaken to ascertain whether the tyrosyl groups of this protein were exposed to the medium. During the course of this investigation, it became evident that treatment of this protein with

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Materials—Bovine \( \alpha \)-lactalbumin was prepared as described previously (20). \( N \)-acetylimidazole was obtained from several commercial sources; trinitrobenzene sulfonic acid was obtained from Pierce Chemical Company, Rockford, Illinois, and hydroxylamine was obtained from Fisher. Glass-distilled water was used throughout.

Acylation and Decylation of \( \alpha \)-Lactalbumin—The procedure for acylation was essentially that of Simpson, Riorden, and Vallee (21) and Riorden, Wacker, and Vallee (22). Solutions containing 0.1 to 1 g/100 ml of \( \alpha \)-lactalbumin in pH 7.0 to 7.00, 0.05 M sodium borate were treated at ambient temperature (20-25°C) with the appropriate concentrations of \( N \)-acetylimidazole. Acylation was carried out at the pH optimum, 7.5, for 1 hour maintaining the pH constant (by addition of aliquots of 1 M KOH) with a Radiometer model 4 pH meter. The reaction mixture was dialyzed overnight against the solvent buffer to remove excess reagents and low molecular weight reaction products. Decapsulation was accomplished by making an aliquot of the acylated protein (in the pH 7.5 borate buffer) 0.2 M in hydroxylamine. In a few instances, decapsulation was carried out at pH 12 (see "Results"). The degree of acylation of tyrosyl groups was determined by both the colorimetric hydrazinolysis procedure (23) and from the change in absorbance at 280 m\( \mu \) on decapsulation (21) as measured with a Cary model 15 spectrophotometer, either as direct or as difference spectra. Decapsulation was complete in about 2 hours. Concentrations of protein were determined from the optical density at 280 m\( \mu \) of the deacylated protein with an extinction coefficient \( E_{280}^{\text{oum}} \) of 20.1 obtained for unreacted protein (1). The justification for this procedure can be seen under "Results." A molecular weight of 14.6 \( \times \) 10^3, as calculated from the amino acid sequence (13-16), was used.

Determination of Amino Groups—Unacylated amino groups in \( \alpha \)-lactalbumin were determined by reaction with \( 1 \text{N} \) \( \text{NBS} \) at room temperature (24). Protein in pH 8.5, 0.05 M borate, 0.05 M phosphate buffer was treated with reagent at a concentration of 50 mg/100 ml. The number of reactive amino groups was obtained from the absorbance at 345 m\( \mu \) with an extinction coefficient of 1.15 \( \times \) 10^4 (24). A reaction time of 3 hours was used since preliminary work with unacylated protein had shown reaction to be complete during this period. The total number of amino groups in unacylated protein was found by this procedure to be 13.1 \( \pm \) 0.1 in good agreement with the amino acid sequence (13-16) which indicates 12 lysines and a single \( \alpha \)-amino group.

Acylated amino groups were determined by measuring the number of amino groups reactive with respect to TNBS after acylation mixtures were freed of excess acetylimidazole and side products by dialysis against the pH 8.5 borate-phosphate buffer used in the TNBS procedure. Protein concentrations were obtained as previously described from the ultraviolet absorption of \( \alpha \)-lactalbumin deacylated with 0.2 M hydroxylamine; in this case, however, this was accomplished at pH 8.5 in the borate-phosphate buffer rather than the usual pH 7.5 borate buffer. The number of tyrosine deacylated was the same in both buffer systems at comparable levels of reagent.

Fluorescence Measurements—Since the instrument used for this work will be described in detail in a later publication, its characteristics are only briefly sketched here. Excitation was with light from an Osram 450-watt xenon lamp dispersed by a Bausch and Lomb 500-mm grating monochromator. Emission spectra were scanned with a second Bausch and Lomb 500-mm monochromator, coupled electronically to one axis of an EAI X-Y recorder. The fluorescent light was detected by an EM1 6256 photomultiplier tube; the resulting signal was amplified by a Princeton JB-4 Lock-in Amplifier, after which it was applied to the second axis of the recorder. The temperature of the cell contents were maintained at 25.0°C by circulating constant temperature water through the jacket integral with the cell compartment. In order to check for changes in lamp intensity and also for use in subsequent quantum yield calculations (see below), the emission spectrum of tryptophan at neutral pH was also scanned. In order to ensure comparable intensities of exciting light, the concentrations of tryptophan were chosen such that their optical densities at the excitation wave length were close to those of the protein solutions. The optical densities for both protein and tryptophan were generally of the order of 0.1. In order to ex sco only tryptophan groups, the excitation wave lengths were restricted to 295 m\( \mu \) (25).

Emission spectra were corrected for photomultiplier-monochromator sensitivity by the use of empirical constants derived from a calibration with a Bureau of Standards quartz-tungsten-iodine lamp (26). Quantum yields were calculated from the emission spectra using the following expression (27).

\[
\frac{Q}{Q_0} = \frac{\int_0^\infty F_d(1 - 10^{-D}) \, dx}{\int_0^\infty F_d(1 - 10^{-D}) \, dx}
\]

The areas under the protein and the tryptophan standard spectra were determined with a planimeter. \( D \) and \( D_t \) are the optical densities of the sample and standard at the excitation wave length.

Circular Dichroism Measurements—CD measurements were made at ambient temperatures with the attachment for the Cary model 60 spectropolarimeter with 1- and 10-mm cells. Protein concentrations and path lengths were chosen to yield optical densities no greater than 1.0 at any wave length. In general, scans were made at the slowest possible speed with the synchronous motor and a time constant of 10 sec. Some difficulty was encountered in reproducibly positioning the base-line after raising and lowering the sample Pockels cell elevator. The stability of the base-line, however, was excellent during the long periods of time required to scan spectra. Periodic remeasurement of ellipticities after the completion of a scan did not reveal changes in general greater than that anticipated from the signal to noise ratio at the wave length in question (see below). Scans of blanks were made before and after those for each protein's solution. In order to compensate for shifts in baseline as cited above, the pen was reset to a fixed position at some wave length above 310 m\( \mu \) where the ellipticity of the protein
solution was zero. Signal to noise ratios were typically: 75:1, 270 mp; 250:1, 230 mp; 200:1, 220 mp; 100:1, 208 to 209 mp.

Additional spectra were obtained with a second Cary instrument through the courtesy of Dr. G. Fasman, Graduate Department of Biochemistry, Brandeis University. These were in good agreement with those measured in our laboratory.

RESULTS

Reactivity of Tyrosyl Groups—Treatment with acetylimidazole indicates differing reactivities of the tyrosyl residues of bovine \( \alpha \)-lactalbumin (Fig. 1). The reaction curve shows a distinct plateau somewhat above 2 tyrosine residues. The third and fourth groups appear to react only with great difficulty and indeed may not represent the reactivity of tyrosyl groups in the native protein (see below).

Tyrosyl reactivity was markedly increased when acylation was carried out in 8 M urea (circles, Fig. 1), showing that the differential tyrosine reactivity observed in the native protein was the result of conformational determinants. The failure to observe complete acylation of the four tyrosyl groups appears to be the result of spontaneous hydrolysis of acyl groups in urea solutions.

Gorbunoff (28) has shown that cyanuric fluoride reacts with all four tyrosine groups at pH 10, where the protein is undergoing a conformational change (9). However, at pH 9.3, below the transition region, a plateau was observed at three reactive tyrosines. Unfortunately, her measurements at this pH were not carried to sufficiently low reagent concentrations to ascertain if differences in reactivity might exist for these three groups. It is of interest to note, however, that the reagent levels required to effect reaction both at pH 9.3 and 10 were markedly higher than those observed with several other proteins that she studied.

Changes in Absorption Spectra on Acylation—With the exception of the two points at low reagent concentration (filled symbols, Fig. 1) the reactivity data shown in this figure were obtained by the colorimetric hydroxamate procedure since the spectral procedure was found to give systematically higher results (average difference approximately one group). The reason for this discrepancy became evident when the spectra of acylated and deacylated \( \alpha \)-lactalbumin were compared as difference spectra (Fig. 2). The relative positions of the acylated and deacylated protein in the sample and reference beams indicate that the spectrum of the latter protein is shifted toward longer wave lengths as compared to the former. The dominant feature

![Fig. 1. Number of tyrosyl residues in \( \alpha \)-lactalbumin acylated as a function of acetylimidazole concentration. O and A, hydroxamate procedure; ▲, spectral procedure.](image1)

![Fig. 2. Difference spectra, deacylated versus acylated protein. The molar ratios of acetylimidazole to protein were as indicated. Measurements made in pH 7.00, 0.05 M borate buffer at ambient temperature (approximately 25°C).](image2)
solution of a-lactalbumin at molar ratios of N-acetylimidazole to protein in excess of 40 results in a rather abrupt change in yield (Fig. 3). This change appears to be complete by a molar ratio of about 60:1, and undergoes no further change over a 3-fold increase of reagent concentration. A comparison of Figs. 1 and 3 (see also Table I) indicates that this increase occurs on acylation of just over 50% of the tyrosyl groups. The increase in yield at high degrees of acylation was more than 3 times that occurring on low degrees of acylation, and the shift in emission maximum was about twice as great. Unlike the spectral changes occurring at low degrees of acylation, those observed at high degrees are not fully reversible on deacylation with hydroxylamine; the quantum yields remain about 30% higher than that of the native protein, and the emission maximum remains 9 

Subsequent to the time that these studies were completed, Chen (29) redetermined the quantum yield of tryptophan relative to that of quinine and found a value of 0.15, which is almost 60% lower than that obtained by Tad and Weber (30). This difference need not be considered here since we are only concerned with changes in yield relative to that of native a-lactalbumin.

In considering reversibility of acylation, we also compared the absolute absorption spectrum of native and acylated a-lactalbumin which had been treated with hydroxylamine. In an experiment with a derivative prepared with a 160:1 molar excess of acetylimidazole, the spectrum obtained with a Cary model 15 spectrophotometer could be normalized to give exact correspondence of spectra for native and deacylated proteins. This instrument is capable of making optical density measurements with a standard deviation of 0.001, where given, are for averages computed for low and high degrees of acylation.

The standard deviations of \( \Delta_{\text{max}} \), where given, are for the averages computed for low and high degrees of acylation; the uncertainty in \( \Delta_{\text{max}} \) for a given spectrum is of the order of 1 to 2 m.

A, acylated protein; \( \Delta \), deacylated protein. Conditions: 25°, solvent pH 7.5, 0.05 M KCl. Acid treated, hydroxylamine; native, conditions as in Fig. 3; acid denatured, and acylated a-lactalbumin. The spectra have been normalized such that the areas are proportional to quantum yield; native, conditions as in Fig. 3; acid denatured protein measured in pH 2.00, 0.15 M KCl.

Examination of the emission spectra for acylated and native a-lactalbumin revealed differences in the wave length distribution of the tryptophan fluorescence at high and low degrees of acylation. The increase in quantum yield occurring for low degrees of acylation appears to be the consequence of changes in emission at the longer wave length, whereas those observed for high degrees of acylation occur in a nonuniform way at both low and high wave lengths. These differences will be clearer from a more detailed analysis of the fluorescence data in the following section.

**Table I**

<table>
<thead>
<tr>
<th>Protein</th>
<th>( Q^2 )</th>
<th>( \lambda_{\text{max}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>0.0580</td>
<td>337</td>
</tr>
<tr>
<td>Acylation, low</td>
<td>0.0071 ± 0.0024</td>
<td>333 ± 2</td>
</tr>
<tr>
<td>Acylation, high</td>
<td>0.0573 ± 0.0039</td>
<td>330 ± 1</td>
</tr>
<tr>
<td>Acid treated</td>
<td>0.0953 ± 0.0028</td>
<td>339 ± 2</td>
</tr>
<tr>
<td>Deacylation, low</td>
<td>0.0692 ± 0.0050</td>
<td>336 ± 1</td>
</tr>
<tr>
<td>Deacylation, high</td>
<td>0.0850</td>
<td>341</td>
</tr>
</tbody>
</table>

*Unless otherwise indicated, the solvent was pH 7.5, 0.05 M borate.*

Yields calculated from Equation 1 with tryptophan as a standard of quantum yield 0.20 (see Footnote 2 and Reference 30). The standard deviations, where given, are for averages computed for low and high degrees of acylation.

A, acylated protein; \( \Delta \), deacylated protein. Conditions: 25°, solvent pH 7.5, 0.05 M sodium borate, unacylated protein measured in pH 6.00, 0.15 M KCl.

![Fig. 3 (top)](image1.png)

**Fig. 3** (top). Tryptophan quantum yields of a-lactalbumin as a function of acetylimidazole concentration. ▲, acylated protein; \( \Delta \), deacylated protein. Conditions: 25°, solvent pH 7.5, 0.05 M sodium borate, unacylated protein measured in pH 6.00, 0.15 M KCl.

![Fig. 4 (bottom)](image2.png)

**Fig. 4** (bottom). Tryptophan emission spectra of native, acid-denatured, and acylated a-lactalbumin. The spectra have been normalized such that the areas are proportional to quantum yield; native, conditions as in Fig. 3; acid denatured protein measured in pH 2.00, 0.15 M KCl.

**Fluorescence Difference Spectra**—Differences in the wave maximum, is about 1000. The corresponding values of \( \Delta_{\text{em}} \) would be approximately 600. Since \( \varepsilon_{280} \) for native protein is 2.90 × 10⁴ (\( \varepsilon_{280} \) equal to 20.1 (1)), molecular weight 14.6 × 10^4 (17-18), the extinction coefficient for deacylated protein, thus, may be as low as 2.84 × 10^4. Use of the extinction coefficient for native protein in determining concentration of deacylated protein, therefore, may involve an error of at most 2%.
length distribution of fluorescent light in the spectra of acylated α-lactalbumin and native protein can be shown readily by calculation of fluorescence difference spectra. The following equation follows from the definition of the quantum yield (27).

\[
\Delta \phi = \left( \frac{F^*}{\lambda} \right)_A - \left( \frac{F^*}{\lambda} \right)_B
\]

(2)

\(F\) is the photocube signal corrected for monochromator and phototube efficiency (see “Experimental Procedure”). \(A\) is the area under the emission spectrum, and \(Q\) is the quantum yield as calculated from Equation 1. The quantity \(\Delta \phi\) is proportional to the difference of the spectral quantum yields (27); it includes, however, a factor which relates the planimeter reading involved in the measurement of spectral areas to the actual area under the curve.

Shown in Fig. 5 is such a difference spectrum calculated for unmodified α-lactalbumin acylated at low levels of reagent. Curve A represents the actual difference spectrum, whereas Curve B is an absolute emission spectrum of tryptophan in water at neutral pH normalized to fit Curve A at wave lengths of 360 mp and above. Since Curves A and B correspond within experimental error, we can conclude that the principal effect of acylation of tyrosyl groups is to increase the quantum yield of 1 or more tryptophan residues whose emission maximum is close to 350 mp.

Similar calculations for unmodified and acylated α-lactalbumin at high levels of reagent yield the curves shown in Fig. 6. In contrast with acylation at low levels of reagent, the difference fluorescence spectrum does not correspond to the spectrum of tryptophan in water. The experimental difference spectrum, Curve A, can be resolved into two components: Curve B, the spectrum of tryptophan in water, and Curve C, the spectrum of tryptophan group having an emission maximum below 320 mp. When one deacylates the protein acylated at high levels of reagent, the form of the spectrum remains essentially unchanged (compare Figs. 6 and 7). Deacylation of O-acyltyrosine groups with hydroxylamine produces a change similar in form, but opposite in sign to that observed on acylation of tyrosine groups at low levels of reagents (Fig. 6). Thus, we can conclude that acylation of α-lactalbumin at high levels of reagent produces two effects: (a) a reversible increase in quantum yield of a tryptophan residue or residues having an emission maximum of 350 mp (this process results from acylation of tyrosyl residues); (b) an irreversible quenching of a low wave length spectral component and enhancement of a component whose maximum is at 350 mp.

It is of interest to note that the fluorescence difference spectrum for acid-treated α-lactalbumin is similar to that observed at high levels of N-acetylimidazole (compare Figs. 7 and 8). In both instances the spectra have a positive 330 mp component and a negative, low wave length emitting component. The significance of this similarity is discussed in subsequent sections of this paper.
Quantum Yields of Tryptophan Groups Perturbed on Acylation—
The resolution of the fluorescence difference spectra carried out in
the previous sections suggests a means for estimating the
quantum yields for individual tryptophan residues which may
have been perturbed on acylation and on acid denaturation.
The fluorescence $F$ and optical density at the excitation wave
length are related in first approximation by the following equation
(27).

$$Q = \frac{\int_0^\infty F \, d\nu}{D} = \frac{KA}{D}$$

where $Q$ is the yield, $A$ is the area under the emission spectrum,
$D$ is the optical density of the protein at the exciting wave
length, and $K$ is a proportionality constant which depends upon
the intensity of the exciting light, the geometry of the spectrom-
eter, etc. Since the spectra were excited at 295 nm, $D_{295}$
is due solely to tryptophan residues. We shall also assume
that the emission from all of the tryptophans of bovine $\alpha$-lact-
albumin occurs independently; i.e. transfer of the excited state
among tryptophan residues does not occur. Although the phys-
ical requirements for transfer of the excited states among the
tryptophans of protein seems to be met, there appears to be
considerable doubt as to whether it actually occurs (see Refer-
ence 31 for a compilation of evidence pro and con). Definitive
evidence for either conclusion seems to be lacking; Elkana (32)
has recently shown that energy transfer among the tryptophans
of lysozyme (a protein in which the relative positions of trypto-
phans should strongly favor transfer) is incompatible with her
observations of energy transfer to salicylamide. Evidence pro
or con for transfer to bovine $\alpha$-lactalbumin is lacking at present.

If we make the assumption that the absorption for each of
the tryptophans in bovine $\alpha$-lactalbumin is the same, we can write
the following expression from Equation 3 for the observed
quantum yield $Q$ in terms of emission from individual trypto-
phan residues.

$$Q = \frac{K}{D} \sum_i A_i$$

but

$$\sum_i Q_i = \frac{K}{D/n} \sum_i A_i$$

where $n$ is the number of tryptophans in the protein molecule.

Hence,

$$Q = \frac{1}{n} \sum_i Q_i$$

If we now designated two states of the protein molecule by
primed and unprimed symbols and neglect changes in $D$ at the
excitation wave length, the change in yield can be written as:

$$\sum_i (Q_i - Q'_i) = \sum_i (Q_i - Q'_j)$$

If the change in yield occurs only for a few groups, it is convenient
to write Equation 7 with a new subscript $j$ designating only
those groups which have been thus influenced by the confor-
mational change.

$$\sum_i (Q_i - Q'_j) = \sum_j (Q_j - Q'_j)$$

The first term on the right hand side of this equation is the sum
of the yields of the groups affected after the conformational
change, whereas the second term represents the sum of the
yields of these groups prior to the structural change. The
yields for groups uninfluenced by such a structural change do
not appear, of course, in the final expression. We can now
estimate the quantum yield of the tryptophan groups which
have been perturbed by acylation and by acid denaturation
making use of Equations 7 and 8. Shown in Column 1 of
Table II are values of $\sum_i (Q_i - Q'_i)$, calculated from the observed
yields (Table I) for native, acylated, deacylated, and acid-de-
natured $\alpha$-lactalbumin. This quantity is of the same order of
magnitude for high acylated and acid-treated protein suggesting,
as did the comparable fluorescence difference spectra (Figs. 6
and 8), that the two processes are similar. Shown also in
Columns 2 and 3 of Table II are $\sum_i Q_i$ and $\sum_i Q'_i$ obtained from
fluorescence difference spectra (Figs. 6-8), assuming that the
negative portions of these curves were proportional to the yields
prior to the structural change and that the positive portions
were proportional to the yields after the structural change. The
values of $\sum_i Q_i$ appear to be the same order of magnitude for
acylated, deacylated, and denatured $\alpha$-lactalbumin, as one
might expect, if the same tryptophan residues were involved in
the conformational changes on acylation and acid treatment.
The large errors which accumulate in this type of calculation
make it difficult to know if the difference in $\sum_i Q'_i$, for acylated,
deacylated, and acid-treated proteins is significant. In any
event, the yield of this critical tryptophan group or groups is
quite low (approximately 0.015 to 0.034) in the native state, as
compared to 0.20 for free tryptophan. It would appear that
these low wave length emitting tryptophans (negative portion
of spectrum, Figs. 6 to 8) are strongly quenched in the native
state. On acylation or acid denaturation, by contrast, they
emit near 350 nm (positive spectral component, Figs. 6 to 8)
and have yields of nearly 0.10 to 0.15, comparable to those of
tryptophan residues incorporated into a peptide chain but in
contact with water.

Circular Dichroic Properties—We have shown previously (3)
4 Quantum yields and emission maxima for tryptophan fluores-
cence show a wide range of variation for native proteins. By
contrast, the emission maxima of proteins denatured in 6 M guan-
idine hydrochloride are uniformly close to 350 nm, and the trypto-
phane quantum yields fall in a much narrower range, 0.10 to 0.15
(see Reference 33). Teale (34) had also shown that the trypto-
phan emission maxima for proteins dissolved in 8 M urea were close
to 350 nm. The fact that virtually the same range of values of
$Q$ is observed after dithiothreitol reduction suggests that this
variation in completely disrupted proteins is due to the influence
of the amino acid sequence per se on the yield (33).
that bovine a-lactalbumin exhibits a complex system of Cotton effects which are quite sensitive to the molecular state of the protein. Changes in the ORD parameter, \(b_0\), which occur on acid denaturation have been shown to be due to virtual elimination of the Cotton effect system occurring in the wavelength region 260 to 320 nm. This earlier study, carried out with a manual spectropolarimeter, also showed that the amplitude of the 233 nm trough remains unchanged on acid treatment. Subsequent measurements made with a Cary model 60 recording spectropolarimeter have revealed small but significant differences in the ORD spectrum in the region of the inflection near 210 nm and in the peak just below 200 nm. Circular dichroism measurements likewise reveal differences in this, as well as in the 245 to 310 nm region, for acid-denatured and acylated a-lactalbumin (Figs. 9 and 10). A preliminary account of the observations for the native and acid-denatured protein has already been given (35) with emphasis on the relationship of its backbone conformation to that of lysozyme. We contrast below the changes in the CD spectrum on acylation with those occurring on acid treatment.

The CD spectrum of native bovine a-lactalbumin is exceedingly complex in the near ultraviolet region (Curve D, Fig. 9). Although the absolute value of the ellipticity is subject to some uncertainty, (see “Experimental Procedure” for discussion of these errors), all of the features seen in the spectra are reproduced in repetitive scans. The spectrum for native protein reveals a small negative band at about 207 nm and a broad negative envelope extending from about 292 to 255 nm which are seen a number of well reproduced inflections and plateaus. It is virtually impossible to resolve unambiguously such a complex spectrum into individual ellipticity bands, but it would appear that it may consist of upwards of six components. By way of contrast, the lysozyme CD spectrum in this wave length region exhibits four relatively well resolved bands: three positive ones at 282, 299, and 295 nm, and a negative one at 290 nm. These results for lysozyme are in general accord with the observations of Ikeda et al. (36) and of Glazer and Simmons (37).

Acylation of a-lactalbumin results in a generalized reduction of the amplitude of the band system (Curves B and C, Fig. 9) without specific alteration of any of the individual bands, as far as can be seen. Like the fluorescence observations, the data fell into two groups: those for low degrees of acylation-molar ratios of N-acetylimidazole of 0 to 40:1, and those for high degrees of acylation-molar ratios of N-acetylimidazole of 60 to 180:1. No significant changes were observed within these two ranges (see average deviation of ellipticities, Table III). Acylation at low levels of reagent resulted in a reduction of nearly 30% of the amplitude of the low ultraviolet CD envelope, whereas high levels of reagent gave a change of about 45% relative to the native protein (Fig. 9, Table III). Deacetylation of tyrosyl residues by treatment with hydroxylamine appeared to reverse the effects of acylation up to about 270 nm at low levels of reagent (unfilled circles, Fig. 9). The deviation of the points from Curve A at lower wave lengths, however, suggests that reversibility may not be total. By contrast, the CD spectrum of hydroxylamine-treated high acylated protein (filled circles, Fig. 9) gave little indication of reversal relative to native protein. It is of interest that the amplitude of the 297 nm peak is somewhat restored; the magnitude of the ellipticity, makes its significance open to question. It is worth noting again that the changes observed on acylation are qualitatively similar.
TABLE III
Circular dichroism of native and acylated bovine α-lactalbumin

The average deviation of values from the mean reflects primarily uncertainty in base-line position (see “Experimental Procedure”); paired measurements (acylated and deacylated protein) always show the reversible increase in ellipticity, although the average deviations seem to suggest no significant differences for the two states of the protein.

<table>
<thead>
<tr>
<th>Protein</th>
<th>λ (nm)</th>
<th>[α] × 10^4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>270</td>
<td>230</td>
</tr>
<tr>
<td>Native</td>
<td>0.275 ± 0.016</td>
<td>9.10 ± 0.58</td>
</tr>
<tr>
<td>Acylated, low*</td>
<td>0.200 ± 0.015</td>
<td>8.49 ± 0.40</td>
</tr>
<tr>
<td>Deacylated, low†</td>
<td>0.295 ± 0.029</td>
<td>8.72 ± 0.33</td>
</tr>
<tr>
<td>Acylated, high‡</td>
<td>0.148 ± 0.018</td>
<td>7.29 ± 0.22</td>
</tr>
<tr>
<td>Deacylated, high‡</td>
<td>0.153 ± 0.030</td>
<td>7.44 ± 0.34</td>
</tr>
<tr>
<td>Acid denatured‡</td>
<td>0.046 ± 0.012</td>
<td>6.43 ± 0.58</td>
</tr>
</tbody>
</table>

* Solvent, pH 7.0, 0.15 M KCl and pH 2, 0.15 M KCl, respectively.  † Average for protein acylated at levels of reagent of 0 to 40:1.  ‡ Average for protein acylated at levels of reagent of 60 to 180:1.

These changes are most readily seen as calculated differences CD spectra (Curve C, Fig. 11). The elimination of the plateau region in the spectrum of the acid protein appears to be the result of the disappearance of a positive ellipticity band centered around 230 nm. The change at 208 to 209 nm is also evident in the different CD spectrum as a positive difference band.

Changes in the far ultraviolet CD spectrum on acylation are qualitatively similar to those observed on acid treatment. The plateau in the region of 213 to 222 nm is largely eliminated at high levels of acylation (Curve B, Fig. 10), and the ellipticity of the negative 208 to 209 nm band increases by about 1000 degrees (see also Table III). This is evident as a negative ellipticity band at 230 nm and a positive ellipticity band at about 208 to 209 nm in the difference spectrum (Fig. 11). The principal change in the spectrum of low acylated α-lactalbumin is in the 208 to 209 nm band (Curve C, Fig. 10), although there is some indication of a small change at the 230 nm difference band (Curve A, Fig. 11). Deacylation does not appear to reverse the change at the 230 nm difference band, but rather strikingly the change at the 208 to 209 nm band is entirely reversible, regardless of the level of acylation.

Acylation of Amino Groups—The lack of complete reversibility of most of the spectral and CD changes after deacylation with hydroxylamine suggested that treatment of bovine α-lactalbumin with N-acetylimidazole may have brought about acylation of groups other than tyrosine. Riorden et al. (22) have shown that thiol and e-amino groups react, to some degree, whereas acylation of the aliphatic hydroxyl residues of serine and threonine were not detectable under the reaction conditions used. In support of the latter observation, we have found that treatment of acylated protein with hydroxylamine at pH 7 and at pH 12, where deacylation of aliphatic OH groups should occur (38), gave identical number of acyl groups by the hydroxamate procedure. Since bovine α-lactalbumin contains no thiol groups, we conclude that amino groups are acylated; this was verified by determination with TNBS (see “Experimental Procedure”).

Treatment of bovine α-lactalbumin with TNBS for 3 hours gave reaction with 13 amino groups in accord with the known sequence. Reaction of TNBS with acylated protein showed that about three amino groups were acylated at the lowest
results in swelling of the bovine casein molecule. The protein, indicating that acylation, like acid denaturation (1, 2), precluded examination of its properties at low pH. Solvent perturbation measurements were made on the samples of acylated bovine casein used in the procedure. For reasons that are not at all obvious, we have been totally unable to reproduce the solvent perturbation observations at 25°. The fact that a tryptophan residue would appear to be exposed to the medium according to solvent perturbation measurements does not necessarily require that its emission maximum correspond to that observed for a free tryptophan residue. Walker, Bednar, and Lumry (40, 41) have shown that the position of the emission maximum for fluorescence of an indole group is not determined primarily by the bulk solvent properties, but is rather a function of the characteristics of the complex formed between the indole molecule in its excited state and the polar solvent molecules, i.e. exciplex formation. For a protein molecule, we would expect the tryptophan to emit at 350 μm only if it were in contact with water molecules and, furthermore, could take on the proper geometry for exciplex formation during the irreversible part of this process appears to correspond to the first phase referred to above, i.e. acylation of the same tyrosyl residue. The irreversible part appears to be the consequence of acylation of an amino group or groups (probably &-amino groups) (Figs. 3, 4, and 12) and gives rise to an increase in yield and an apparent long wave length shift of the spectrum. In this case, the difference fluorescence spectrum (Fig. 6) indicates quenching of tryptophan emitting below 320 μm and an increase in the yield of tryptophan emitting at 350 μm. Two alternative explanations of this observation can be made. (a) Acylation of amino groups may induce a structural change which quenches the fluorescence from a buried tryptophan (the low wave length emitting group) without exposing this group and which also increases the yield of a second tryptophan, which is already freely in contact with water molecules, e.g. the same tryptophan residue whose yield is increased on acylation of a tyrosyl group. (b) Acylation of amino groups results in a structural change which alters the environment of a highly quenched low wave length emitting tryptophan which is not a buried group so as to bring it into free contact with water molecules (see below for the meaning of “free”). The possibility of a change being the result of exposure of a previously buried tryptophan is excluded by the observation that no enhanced exposure occurs on acylation.

Our data do not permit us to unambiguously distinguish between these two alternative explanations. The second explanation, however, is perhaps the simpler one.

The fact that a tryptophan residue would appear to be exposed to the medium according to solvent perturbation measurements does not necessarily require that its emission maximum correspond to that observed for a free tryptophan residue. Walker, Bednar, and Lumry (40, 41) have shown that the position of the emission maximum for fluorescence of an indole group is not determined primarily by the bulk solvent properties, but is rather a function of the characteristics of the complex formed between the indole molecule in its excited state and the polar solvent molecules, i.e. exciplex formation. For a protein molecule, we would expect the tryptophan to emit at 350 μm only if it were in contact with water molecules and, furthermore, could take on the proper geometry for exciplex formation during the lifetime of the excited state. We would consider a tryptophan in a protein, capable of forming an excited state complex with water molecules, as being freely in contact with water. Restricted rotation of such a tryptophan in a crevice-like region of the molecule, for example, might prevent complex formation with water molecules or with other polar side chain moieties. A more detailed account of this mechanism has been given earlier (7). Eisinger and Navon (42) have recently suggested that the effect of the solvent environment on the wave length distribution of fluorescence from tryptophan is better explained in terms of preferential solvation, rather than formation of discrete excited state complexes. Either point of view, however,
leads to the same conclusion in explaining the dependence of the fluorescence properties of a tryptophan residue in a protein on its freedom of rotation in a polar environment.

Changes in Circular Dichroism Spectra—The changes in CD spectra, which must be accounted for in this study, are: (a) the small reversible change in the 208 μm negative ellipticity band occurring on acid denaturation and on acylation at low levels of reagent (Fig. 10); (b) the elimination of a positive 230 μm ellipticity band (Fig. 11) occurring on acid denaturation and on acylation (primarily at high levels of reagent), in which ε-amino groups show enhanced reactivity (Fig. 12); and (c) the virtual elimination of the 250 to 300 μm band system on acid denaturation, its essentially reversible reduction in amplitude at low degrees of denaturation, and the irreversible reduction at high degrees of denaturation (Fig. 9). These changes are accountable in principle in terms of: (a) optically active peptide bond transitions which are determined by the conformation of the polypeptide backbone, e.g., presence of helical, β, random structures (see Reference 43 for a recent summary of the rotatory properties of these conformations); and (b) transitions involving side chains such as tyrosine, cystine, and tryptophan. Because of the complexity of these spectra and a paucity of basic information concerning the molecular determinants of the sign and position of the various bands in proteins there has been little success in general in making unequivocal assignments of ultraviolet CD bands. Since there is reason to believe that side chain bands may occur at relatively short wave lengths (see below), overlap with peptide band transitions may subject any calculation of the amount of ordered conformations in a protein molecule to considerable uncertainty. We do not concern ourselves here with the more fundamental question as to whether protein molecules can be arbitrarily considered to be made up of regions of α helical, β, and random conformations.

In spite of this uncertainty in making band assignments and in estimating the contributions of various kinds of ordered conformations to the protein structure backbone, some general conclusions can be made concerning changes in the CD properties of α-lactalbumin on acylation or acid treatment. It is apparent from the magnitude of the changes in the low ultraviolet CD spectrum (Fig. 10) (see also Fig. 1 in Reference 35 for the CD spectrum of native and acid-denatured α-lactalbumin below 200 μm) that neither acylation nor acid denaturation results in large scale alterations of the backbone conformation of the α-lactalbumin molecule. The possibility exists, of course, that changes in CD spectra resulting from alterations of backbone conformation are exactly compensated by changes due to side chain optical activity. Indeed, it is difficult to envision any conformational change occurring without alteration of some of the polypeptide backbone of the protein molecule.

The CD spectrum of native α-lactalbumin in the region of 200 to 300 μm is exceedingly rich in detail (Fig. 9). The maximum value of the ellipticity is rather large, comparable to that observed with human carbonic anhydrase (44), avidin (45), and pancreatic trypsin inhibitor (46), but considerably larger than that found for β-lactoglobulin (47) and for lysozyme (35–37, 46). It is impossible, however, to make definite assignments of the various bands making up this complex system below 300 μm (Fig. 9). Although acylation of tyrosyl groups has been shown to alter the fluorescence properties of tryptophan, it is not obvious whether any of the changes that occur in the 250 to 300 μm band system are due to alteration of the environment of tyrosine groups per se. It is quite evident from studies with polypeptides that tryptophan residues must make a significant contribution to the CD spectrum in this wave length region.

The CD spectrum of poly-L-tryptophan (48) is rather complex in the wave length region of 250 to 300 μm; it consists of a broad positive envelope in which several bands are resolved. The longest wave length band occurs just above 290 μm. Since a similar long wave length band occurs in native α-lactalbumin (approximately 293 μm) (Fig. 9), it seems likely that this band, as well as much of the broad envelope observed at lower wave lengths, must be due to tryptophan residues. Thus, reduction of this ellipticity band system on acylation or acid denaturation must be due at least in part to alteration of the environment of tryptophyl residues, a conclusion supported by changes observed in absorption and emission spectra.

As we have shown, the increase in slope of the CD spectrum in the 213 to 222 μm region (Fig. 10) occurring on acid denaturation and on acylation (primarily at high levels of reagent) is due to elimination of a negative ellipticity band lying below 230 μm (Fig. 11). Poly-L-tyrosine, N-acetyltyrosine ethyl ester, and N-acetylserylamine amide all exhibit a positive band lying between 222 and 224 μm (45). It is doubtful whether the change in the 230 μm band in α-lactalbumin is due to acylation of tyrosyl groups per se since the magnitude of the ellipticity for the 225 μm band of N-acetyltyrosine amide decreases by only 20% on O-acylation (45). The lack of reversibility of this change in CD on treatment with hydroxylamine is in accord with this conclusion. Changes in the perturbation of tyrosine groups occurring as a result of the conformational change induced by acylation of amino groups, however, cannot be excluded.

N-Acetyltryptophan ethyl ester and tryptophan in neutral and acid solution show a positive band lying between 222 and 226 μm whereas in basic solution the tryptophan band lies at 227 μm (46). Poly-L-tryptophan film exhibits a negative band at 232 μm which overlaps the peptide band. On the other hand, Peggion et al. (48) have found a positive CD band at 230 μm for poly-L-tryptophan in ethylene glycol monomethyl ether, whereas block copolymers of L-tryptophan and α-ethyl α-methylglucamide in trifluoroethanol exhibited a positive band at 226 μm (49). Thus, it appears that the 230 μm difference band (Fig. 11) observed on acylation or acid denaturation of α-lactalbumin could be accounted for on the basis of changes in the perturbation of tryptophan groups (see below). Finally, we must consider the small changes in ellipticity of the 208 μm band occurring in acid denaturation and acylation (Fig. 10, Table III). The magnitude of this change within experimental error is the same for both acid treatment and for high and low acylation. Furthermore, decylation of tyrosyl groups with hydroxylamine appears to reverse the CD change within experimental error. Thus, we would expect that changes in the ellipticity in the 208 μm band are the result of changes in the composition of the α-lactalbumin molecule. The possibility exists, of course, that changes in CD spectra resulting from alterations of backbone conformation are exactly compensated by changes due to side chain optical activity. Indeed, it is difficult to envision any conformational change occurring without alteration of some of the polypeptide backbone of the protein molecule.

In an earlier paper (5) we considered the nature of the change in environment of polypeptides that tryptophan residues might result in abolition or reduction of side chain Cotton effects. Little further can be added to the conclusions reached earlier,
namely that the conformational change that bovine \( \alpha \)-lactalbumin undergoes in acid solution results in increased freedom of rotation of side chain chromophores such as tryptophan. The increased freedom of rotation in the acid-treated or acylated protein might be expected to eliminate interaction of such groups with other structural moieties which give rise to enhanced rotational strengths in the native state.

Environment of Tryptophans in Bovine \( \alpha \)-Lactalbumin—Although no definitive information is available at present concerning the actual three-dimensional structure of bovine \( \alpha \)-lactalbumin, it will be useful to consider the environment of tryptophan residues as seen in the molecular model proposed by Browne et al. (17) based on the similarity in amino acid sequence of hen egg white lysozyme and bovine \( \alpha \)-lactalbumin. On initial evaluations of the molecular model were made in the laboratory of Dr. R. L. Hill, Biochemistry Department, Duke University Medical School, to whom we are indebted for this opportunity. More complete observations have been made more recently on a model constructed by Dr. M. J. Kronman at the Upstate Medical Center from lysozyme atomic coordinates generously supplied by Professor D. C. Phillips and following the recommendations of Browne et al. (17). In subsequent discussions we follow the procedure of the latter authors and refer to the bovine \( \alpha \)-lactalbumin sequence positions by the corresponding lysozyme residue number. The number in parentheses gives the actual sequence position in bovine \( \alpha \)-lactalbumin.

Although recent low angle x-ray scattering data for lysozyme and bovine \( \alpha \)-lactalbumin suggest dissimilar shapes in solution (50), other solution properties seem to be in accord with the model proposed by Browne et al. (17). A definitive answer will clearly be forthcoming only when the x-ray crystallography of the protein is completed. Evidence for the similarity includes the following. ORD (51) and CD (19) measurements suggest a similarity in backbone conformation of the two proteins. Infrared spectra provide evidence for comparable amounts of the extended conformation in both lysozyme and \( \alpha \)-lactalbumin (52), and high resolution nuclear magnetic resonance spectra of the latter protein (52) are in semiquantitative agreement with predictions made from the model proposed by Browne et al. (17). Castellino and Hill (53) have recently shown that the relative rates of carboxymethylation of His-35 (BAL 22), 71 (BAL 68), 111 (BAL 107), and Met-93 (BAL 90) are in accord with the environments of these groups as seen in the model. Measurements of solvent perturbation (4) and chemical reactivity of tryptophans are likewise in accord with the model. We have shown earlier that slightly less than two groups were exposed in both the native (pH 6) and acid-denatured (pH 2) protein with no dependence of exposure on perturbant size at 25°. Calculation of exposure of groups in these previous studies were made on the basis of an amino acid content of 5 tyrosine and 5 tryptophan residues. The number of exposed groups computed on the basis of four tyrosines and four tryptophans (13-16) is slightly less than two. We have recently reinvestigated the solvent perturbation properties of bovine \( \alpha \)-lactalbumin with the use of a Cary model 16 spectrophotometer. The observations made with glycerin, ethylene glycol, and sucrose at pH 2 and 6 at 25° are essentially identical with the earlier observations. Castellino and Hill (54) have shown that 1.7 tryptophan residues of \( \alpha \)-lactalbumin react with N-bromosuccinimide at low levels of reagent (reagent to protein molar ratio, 8:1). Of the 4 tryptophans in \( \alpha \)-lactalbumin, only 1, Trp-28 (BAL 26), can be regarded as definitely buried, i.e. surrounded by hydrophobic residues, including Leu-17 (BAL 15), Leu-99 (BAL 96), Leu-3 (BAL 3), Val-8 (BAL 8), Leu-12 (BAL 12), Leu-55 (BAL 52), Phe-56 (BAL 53), Leu-88 (BAL 85), Leu-92 (BAL 89), and Trp-108 (BAL 104). Of the 4 tryptophans, residue 123 (BAL 118) appears to be the most exposed, lying on the surface of the molecule with little hindrance from other side chains being apparent. Trp-108 (BAL 104) and 63 (BAL 60) appear to be less exposed. These residues, together with Tyr-107 (BAL 103), lie in a cleft-like region of the molecule homologous with the active site cleft of lysozyme (Fig. 13). Trp-108 is somewhat hindered by Tyr-107 and by side chains on the lower right-hand side of the cleft. Trp-63 seems less accessible than -108, being surrounded by Ile-58 (BAL 55), Leu-75 (BAL 72), Ile-98 (BAL 95), Ile-62 (BAL 59), and Tyr-107 (BAL 103). The proximity of disulfide bridge 76-94 (BAL 73-92) might also be expected to shield Trp-63 from contact with the medium (see for example solvent perturbation observations made with denatured proteins with intact disulfide bridges, Reference 55). The gradation in apparent exposure seen in the molecular model is consistent with the chemical reactivity studies of Castellino and Hill (54); i.e. residues 108 and 123 are reactive, whereas 28 and 63 are not. Since one cannot expect an exact correspondence between solvent perturbation and chemical reactivity studies (see Reference 55 for discussion of this point), we cannot exclude the possibility that both Trp-63 and -108 might be seen in solvent perturbation as partially exposed groups.

Environment of Tyrosyl Groups—We have shown that two
tyrosyl groups of bovine \(\alpha\)-lactalbumin react poorly with acetyl-
imidazole (Fig. 1). In the model cited in the previous section, however, all 4 residues appear to be on the surface of the molecule with Tyr-107 (BAL 103) perhaps being an exception. The proximity of this residue to Trp-63 and -108 (Fig. 13) would probably shield it from intimate contact with the medium. Studies of nitrations with tetrinitromethane, however, indicate that Tyr-107 is one of the most reactive residues (56). A preliminary published account (57) of a study of the reaction of tetrinitromethane with bovine \(\alpha\)-lactalbumin indicates only 2.5 reactive tyrosines, but in agreement with the studies of Robins, Holmes, and Andretti (54) it also shows reaction with tryptophans. The hydrogen-bonded state of the phenolic group of tyrosine in proteins can apparently determine its chemical reactivity. In the case of lysozyme, a single residue ionizes more slowly than the others (58, 59) and is unreactive with \(N\) acetylimidazole\(^7\) with tetrinitromethane (57) and with iodine (60). Both the iodination and nitrations studies show that the reactive residue in lysozyme is Tyr-53 which is hydrogen bonded in the antiparallel pleated sheet region (18). Since the hydrogen-bonding pattern probably remains unchanged in the homologous extended \(\beta\) region of the \(\alpha\)-lactalbumin molecule (92), we might expect that Tyr-53 would likewise be unreactive. Conformation of this hypothesis will require direct verification.

According to the model, all 4 tryptophans of bovine \(\alpha\)-lactalbumin are relatively close to tyrosyl residues (Fig. 13). The nature of the change occurring on acylation of a tyrosyl group, however, is such that only the pairs Tyr-38 (BAL 36)-Trp-123 (BAL 118), and Tyr-107 (BAL 103)-Trp-108 (BAL 104) would likely have the requisite molecular properties, i.e. free exposure of the tryptophan residues to the medium.

**Tryptophan Groups Perturbed on Acylation of Amino Groups**—Inspection of the model reveals no specific amino groups close enough to tryptophyl residues to directly perturb them on acylation. The change in properties of \(\alpha\)-lactalbumin on acid treatment or on acylation of amino groups appears to be the consequence of a conformational change of a rather local nature (see following section). It is thus quite difficult to pinpoint specific e-amino groups involved in perturbation of the tryptophans. As indicated above, two alternative explanations can be given for the change in fluorescence properties, i.e. disappearance of a low wave length emitting component from the spectrum and enhancement of a component emitting maximally at 350 \(\mu\)m (see Fig. 7). The first explanation involves quenching of a low wave length emitting residue and enhancement of the fluorescence of a 2nd tryptophan emitting at 350 \(\mu\)m. The environment of Trp-28 and -63 (see above) makes them likely choices for the low wave length emitting residue or residues. The long wave length emitting residue could be either Trp-123 or Trp-108. The second explanation involves conversion of a short wave length emitting residue into one emitting at 350 \(\mu\)m. It seems improbable that Trp-28 is the short wave length emitter since such a conversion would require exposure of this group; this has been ruled out by solvent perturbation measurements. The most likely choice is Trp-63, which projects out into the cleft (see Fig. 13) and appears insulated from good contact with solvent by the proximity of a number of side chains (see Fig. 13 and previous discussion of environment of tryptophan residues).

\(^7\) F. M. Robbins and L. G. Holmes, unpublished experiments.

**Nature of the Conformational Changes**—As we have emphasized, changes in the tryptophan emission and CD spectra on acylation of amino groups are quite similar to those observed on acid denaturation. In both instances the apparent shift of the emission spectrum to longer wave length and the increase in quantum yield are the result of a disappearance of a short wave length component and the enhancement of a 360 \(\mu\)m emitting component (see Figs. 7 and 8). Both the acid-denatured (2) and acylated protein have increased frictional ratios. Acid-treated and acylated protein exhibit the same kind of changes in CD spectra both in the high (Fig. 9) and low (Fig. 10) ultraviolet regions. In quantitative terms, however, the changes observed on acid treatment, acylation, and alkaline denaturation are not identical. The change in quantum yield on acylation of only e-amino groups is lower than that for acid denaturation (Column 1, Table II; compare values for acid-treated and acylated, high, deacylated \(\alpha\)-lactalbumin). Changes in the amplitude of the 290 to 300 \(\mu\)m CD band system are smaller on acylation than on acid treatment (Fig. 9). ORD measurements likewise indicate that the changes in the side chain Cotton effects are smaller at alkaline pH than on acid treatment (8). These observations have been recently confirmed by CD measurements.\(^7\) In spite of these quantitative differences, it would appear that similar conformational changes can be brought about by titration of carboxyl groups, or reaction with 1 ethyl-3-(3 dimethylamino propyl) carbodiimide and glycaminide (61), amidination of e-amino groups (6) and, as seen in this study, acylation of e-amino groups. The fact that no large changes in the CD spectra are seen in the far ultraviolet region (Fig. 10) indicates that no gross alteration of the peptide backbone folding occurs, and that the change in conformation must be rather local in nature.

The fact that both carboxyl and e-amino groups are involved suggests that breaking of charge pairs may initiate such a local conformational change, and, indeed, inspection of the molecular model of bovine \(\alpha\)-lactalbumin does reveal a number of potential charge pairs. Browne et al. (17) have pointed out that the following charge pairs may be less than 5 to 6 \(\AA\) apart in BAL: Lys-13 (BAL 13)-terminal carboxyl (Leu-129); Lys-97 (BAL 97)-Asp-101 (BAL 97); Lys-18 (BAL 16)-Asp-102 (BAL 102); Lys-82 (BAL 79)-Asp-81 (BAL 78); Lys-118 (BAL 114)-Glu-117 (BAL 113) or Asp-120 (BAL 10)-Glu-11 (BAL 11). All of these interacting groups with the exception of Lys-82-Asp-81 are located on the right-hand wing of the molecule (Fig. 13), a large number being relatively close to the critical tyrosine and tryptophan residues (see previous sections). We believe that the integrity of some of these charge pairs may be requisite for maintaining the local conformation and that their titration or chemical reaction may be sufficient to destabilize the molecule in that region. The involvement of the pair, Arg-10-Glu-11, might explain the more extensive character of the conformational change produced by low pH treatment as compared to acylation of e-amino groups or high pH treatment (see above), since treatment with acetylimidazole would have no effect on arginine and since such a group would not be titrated at pH 12.

The probable involvement of Trp-63 in the conformational change (see above) suggests that alterations of side chain-side chain interactions may occur in the upper area of the cleft (Fig. 13), a region rather rich in hydrophobic side chains (see "Environment of Tryptophans in Bovine \(\alpha\)-Lactalbumin"). Alteration
of the mutual interactions of such side chains might explain changes in the hydrophobic character of acid-treated bovine α-lactalbumin as evidenced by alteration of the fluorescence of liganded 1,8-anilino naphthalene sulfonic acid, as well as a pronounced tendency of the protein to aggregate at acid and alkaline pH (1, 2). It is of interest to note that lysozyme also shows a marked tendency to associate and that the site of interaction appears to be the region of the active site cleft (62) homologous with the cleft of bovine α-lactalbumin (Fig. 13).

It is rather tempting to conclude that such a hydrophobic site on α-lactalbumin is the binding site for the A protein (the galactosyltransferase) of lactose synthetase; the kinetic data of Castellino and Hill (53) indicate that such an interaction is likely. The loss of activity occurring on carboxymethylation of His-35 and -71 (53) and on oxidation of Trp-63 and -123 (see Reference 52) and their position in the cleft region (see Fig. 13 for location of tryptophan groups) is likewise consistent with this hypothesis. If this proves to be the case, the conformational flexibility of the cleft region of α-lactalbumin (as implied by observations at acid pH and on acylation) would likewise facilitate interaction with the galactosyltransferase. The verification of this hypothesis will require direct studies of the interaction of Component A with α-lactalbumin. Such investigations are currently being undertaken by Dr. M. J. Kronman at Upstate Medical Center.

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