Acetylation of Amino Groups and Its Effect on the Conformation and Immunological Activity of Ovalbumin*

(Received for publication, April 4, 1974)

AFTAB A. ANSARI,† SHOAIB A. KIDWAI,§ and AHMAD SALAHUDDIN¶

From the Department of Biochemistry, Jawaharlal Nehru Medical College, A. M. U., Agra 202001, India

SUMMARY

A procedure is described for the specific acetylation of the lysine residues of ovalbumin. Six acetylated ovalbumins varying in the degree of modification from 21 to 98% were prepared and were found to be homogeneous by polyacrylamide gel electrophoresis, immunodiffusion, and immunoelectrophoresis. As expected, the anodic movement of ovalbumin increased and the isionic point shifted to lower pH values with progressive acetylation of the protein. Measurements on ultraviolet absorption, fluorescence, tryptic digestion, intrinsic viscosity, gel filtration behavior, and immunological reactivity demonstrated that the native folded conformation of ovalbumin was appreciably altered by acetylation. However, even the maximally modified ovalbumin retained considerable residual structure consisting of regions of ordered structure containing antigenic determinants.

The biological role of lysine residues in several enzymes (1) has been demonstrated by chemical modification techniques. However, the conformational implications of such lysine modification thus far have not been properly understood. For example, succinylation of amino groups, which changes positive charges into negative ones, produces major conformational changes in some proteins (2-4) but does not affect the conformational integrity of others (5). Further, carbamylation and acetylation may or may not cause protein unfolding (4, 6). Therefore, for the same reagent, the extent of disruption of native protein conformation by modification of lysine may be different for different proteins. In fact, the disrupting influence of lysine modification depends on the folded stability of a particular protein and on how necessary the lysine residues are for the formation of ordered structures.

The contribution of a lysine residue to the thermodynamic stability of a native protein conformation may principally arise from hydrophobic interactions involving its four CH$_2$ groups and from hydrophilic interactions in which the e-amino group participates directly. It is the latter which is likely to be altered by chemical modification. How do such modifications alter the physicochemical properties of proteins in solution and thereby induce conformational transitions? A systematic study of the properties of native and modified proteins is a prerequisite to understand the relationship. In this paper we report one such study on ovalbumin and its six acetylated forms. The system is suitable to examine the effect of the absence of hydrophilic interactions on the physicochemical behavior and hence on the structural organization of the protein in solution. We have shown that acetylation of e-amino groups produces definite conformational changes in ovalbumin which have a marked deleterious effect on its immunological activity.

EXPERIMENTAL PROCEDURE

Materials

Crystalline ovalbumin was either purchased from Sigma Chemical Co. (St. Louis, Mo.) or isolated from egg white by the method of Kekwick and Cannan (7). Both preparations were identical and free from extraneous proteins as judged by Sephadex gel filtration, CM-cellulose column chromatography, polyacrylamide gel electrophoresis, sodium dodecyl sulfate polyacrylamide gel electrophoresis, and immunological techniques. Cytochrome c, 6-cholesterol, trypsin, bovine serum albumin, and N-acetyltryptophanamide were all Sigma products. Acetic anhydride, propylene glycol, bromophenol blue, and ninhydrin were supplied by BDH Chemicals Ltd. (Poole, England). Hydridantin was prepared according to a standard procedure (g) and methyl Cellosolve was purchased from E. Merck (Darmstadt, Germany). Acrylamide, riboflavin, Amido schwartz (E. Merck), N,N'-methylenebisacrylamide, N,N',N"-tetramethylethylene diamine (Fluka, Switzerland), and ammonium persulfate (Riedel-De Haen AG, Seelze Hannover, Germany) were used in polyacrylamide gel electrophoresis. Sephadex G-100 and Blue Dextran 2000 were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Bacto-agar was supplied by Difco Laboratories (Michigan). Other chemicals were of reagent grade.

Methods

The studies described in this paper were carried out in sodium phosphate buffer, pH 7.0, ionic strength 0.15, unless stated otherwise.
Acetylation of Ovalbumin—Among the various reagents tried in the acetylation of proteins, acetic anhydride is considered to be the reagent of choice (9). Two procedures for the acetylation of ovalbumin were employed. The first method, which involved the treatment of the protein with excess acetic anhydride in half-saturated sodium acetate (pH 8.0), was found to be inadequate, because acetylation invariably led to irreversible protein precipitation. The procedure to be described below was therefore used.

Ovalbumin (200 mg) was dissolved in 20 ml of 0.09 m sodium phosphate buffer, pH 7.5, in a reaction vessel kept at 0-4°C with constant stirring. Acetic anhydride was added in small aliquots and the pH of the reaction mixture was maintained between 7.4 and 7.6 by the addition of 1 N NaOH. The reaction was carried out for 30 min, after which the solution was extensively dialyzed against half-saturated sodium acetate, distilled water, and finally against several changes of phosphate buffer, pH 7.0, ionic strength 0.15. The solution and the final dialysate were passed through Millipore filters and stored in a freezer. The possible O-acetylation of the tyrosine residues of ovalbumin was checked by treating the acetylated protein with 1 m hydroxylamine at pH 7.5 (9).

Ninhydrin Reaction—The ninhydrin reaction was carried out essentially by the method of Moore and Stein (8). After the reaction, the colored product was diluted with 50% (v/v) propanol and 50% (v/v) ethanol. The per hour decrease in optical density in the four cases was 20, 12, 4, and 3%, respectively. Obviously, 50% (v/v) ethanol seems to be the best. The ninhydrin reaction demonstrated the inadequacy of water as the diluent which had been described by Paik and Kim (10).

Quantification of Modification—The extent of the modification of lysine residues in ovalbumin was determined by the ninhydrin method from the plot of optical density at 570 nm versus the concentration, c, in grams per ml, was charged into the viscometer and its times of fall, t, measured at 25°C ± 0.1°C with a stopwatch reading to 1/10 s. The time of fall for the solvent, t₀, was measured similarly, the density of the solvent, ρ₀, was measured by standard procedures. The intrinsic viscosity, [η], was computed from a plot of (t-t₀)/t₀ versus c obtained by the method of least squares according to the relation (17):

\[
[\eta] = \frac{1}{c} \int_{c_0}^{c} \frac{1}{c} dc + \frac{1}{c_0} \int_{c_0}^{c} \frac{1}{c} dc
\]

where c₀ is the partial specific volume of the protein and was taken to be 0.48 ml/g for native as well as modified ovalbumins. The error involved in the measurement of t and t₀ varied between 0.01 and 0.04%, which would introduce a maximum uncertainty of 2.5% in the evaluation of intrinsic viscosity.

Tryptic Hydrolysis—Tryptic digestion of native and modified ovalbumins was performed at pH 7.5 and 33°C according to Paik and Kim (10).

Spectral Measurements—Ultraviolet spectral measurements were made in 1 cm eilea cells either with a Beckman model DK 2A ratio recording spectrophotometer or with a Carl Zeiss spectrophotometer, model VSU-2-P. Scan speed and time constant were chosen to maintain adequate reaction time and to allow adequate signal to noise ratios. The absorbance throughout the scanning range was below the limit where stray light could affect the results seriously.

Fluorescence Measurements—The excitation and emission fluorescence spectra of the proteins were recorded with an Aminco-Bowman spectrophotofluorometer. Fluorescence perturbation measurements were made according to Steiner et al. (18).

Immunological Techniques—Rabbit antiserum against native ovalbumin was raised by injection of alum-precipitated ovalbumin as described earlier (19). The antiserum was diluted 2 times with 0.15 M NaCl and heated to 56°C for 30 min to inactivate the complements prior to use. Double diffusion and immunelectrophoresis were performed according to standard procedures (20). Quantitative precipitin titrations were carried out by a slight modification of the method of Heidelberger and Kendall (21). Increasing amounts of ovalbumin (native or modified) were added to 1 ml of the antiserum and incubated for 1 hour at 45°C (19) followed by incubation at 4°C for 20 hours. The precipitates were washed twice with 10 ml of cold 0.9% NaCl solution (saline) and finally dissolved in 5 ml of KCl-HCl mixture, pH 2.2, ionic strength 0.06 (22). The optical density was recorded at 280 nm.

RESULTS

Extent of Modification—In general, treatment of proteins with acetic anhydride results in the rapid modification of amino and thiol groups, followed by relatively slower modification of tyrosine, serine, threonine, and possibly histidine residues (23). All these modifications, except those of lysine residues, are reversible (23).

Under the conditions of our experiment only lysine residues of ovalbumin would be modified, since high concentration of acetic acid would hydrolyze O-acetyl-L-lysyl residues. The α-amino group in ovalbumin is N-acetylated (24). The tyrosine phenolic groups that are inaccessible (25) in native ovalbumin would become available for reaction with acetic anhydride upon protein denaturation induced by chemical modification. However, we found that the increase in absorbance at 278 nm upon treatment of the acetylated ovalbumins with 1 m hydroxylamine at pH 7.5 (9) corresponded to the modification of only 0.7 or even less tyrosine residues.

**Fig. 1** illustrates the N-acetylation of the free amino groups in ovalbumin caused by treatment of the protein with incremental amounts of acetic anhydride. The extent of modification is given in Table I, in which each value for the per cent of groups modified represents an average of 10 determinations with a precision of 2%. Evidently, the extent of chemical modification was higher at higher acetic anhydride concentration.
The increase in the extent of modification leads to a definite decrease in the point of zero net proton charge. This is quite understandable because acetic anhydride converts the positively charged e-amino groups of ovalbumin to neutral groups.

**Effect of acetylation on isoinic pH, relative mobility, and extinction coefficient of ovalbumin**

<table>
<thead>
<tr>
<th>Molar ratio of anhydride to protein</th>
<th>e-NH₂ groups modified</th>
<th>Isionic point</th>
<th>Relative mobility</th>
<th>Molar extinction coefficient (\times 10^{-4})</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>4.9</td>
<td>0.28</td>
<td>30.1</td>
</tr>
<tr>
<td>11</td>
<td>21</td>
<td>4.6</td>
<td>0.33</td>
<td>29.5</td>
</tr>
<tr>
<td>22</td>
<td>28</td>
<td>4.5</td>
<td>0.34</td>
<td>29.0</td>
</tr>
<tr>
<td>88</td>
<td>62</td>
<td>4.2</td>
<td>0.57</td>
<td>27.9</td>
</tr>
<tr>
<td>110</td>
<td>66</td>
<td>4.2</td>
<td>0.57</td>
<td>27.2</td>
</tr>
<tr>
<td>880</td>
<td>93</td>
<td>4.1</td>
<td>0.54</td>
<td>21.2</td>
</tr>
<tr>
<td>1100</td>
<td>98</td>
<td>4.1</td>
<td>0.55</td>
<td>20.3</td>
</tr>
</tbody>
</table>

\(a\) Electrophoretic mobility with respect to that of bromphenol blue.

\(b\) Molar extinction coefficient, \(c_{\text{M}}\), was determined in phosphate buffer, pH 7.0, ionic strength, 0.15.

The electrical mobility of a protein in gel depends, among other factors, on the net charge and size of the protein molecule (27). Other conditions being the same, the mobility should vary linearly with charge in the absence of conformational changes. The net charge on ovalbumin near pH 7.0, at which the gel electrophoresis experiments were performed, was calculated from the results on the hydrogen ion titration of ovalbumin studied by Cannan et al. (25) and was \(-12\) under our experimental conditions. The net charge on the modified ovalbumin, with all its lysine residues acetylated, would be \(-31\). It can be seen in Table I that the increase in the mobility with increase in the net negative charge is linear only up to 28% modification and becomes nonlinear for the higher modifications.

**Tryptic Digestion**—Following Klee (28) we have used susceptibility of ovalbumin and its various acetylated forms to trypsin digestion as a conformational probe. The time course of the trypsin cleavage of native and modified ovalbumins is shown in Fig. 3, where \(\Delta A_{280}\) represents the net increase in the intensity of the ninhydrin color due to e-amino groups that were newly formed as a result of proteolysis. The average error in the values of \(\Delta A_{280}\) was generally about 0.5%.

Since trypsin cleaves only those peptide bonds in proteins whose carbonyl groups are contributed by arginine and lysine residues and acetylation makes the lysine peptide bonds refractory to trypsin attack (29), \(\Delta A_{280}\) would provide a measure of the exposure of the arginine peptide bonds in the modified ovalbumins. In agreement with a previous report (30) we found...
that native ovalbumin is resistant to trypsin attack (see Fig. 3). Further, for various degrees of modification of lysine residues of ovalbumin, proteolysis was extensive for 93 to 98%, moderate for 62 to 66%, small for 28%, and undetectable for 21% of modified protein.

Spectral Properties—The ultraviolet absorption changes of ovalbumin caused by acetylation are illustrated in Fig. 4; acetylation produced a significant decrease in the absorbance, which became more pronounced with increase in the extent of modification. For 98% acetylated ovalbumin, the decrease at 280 nm was as high as 33% (see Table I). The absorption maxima for 93% and 98% acetylated ovalbumins showed a small but detectable blue shift. The difference spectra of 98% acetylated ovalbumin against the native protein showed the following important features: a small shoulder at 293 nm and negative peaks at 286.5, 281, and 272 nm. Since, in most of the proteins, tryptophan and tyrosine peaks occur, respectively, near 293 and 287 nm, the shoulder at 293 nm and the trough at 286.5 nm represent, respectively, the perturbation of tryptophan and tyrosine spectra. The acid difference spectra studied first by Yanari and Bovey (31) and reinvestigated here showed all the fine structures which were found for 98% acetylated ovalbumin. That ovalbumin undergoes acid denaturation has been shown earlier by Charlwood and Ens (32) and by Ahmad of this laboratory. Thus, the results on ultraviolet absorption changes indicate unfolding of the modified ovalbumins. It should be noted that the 33% decrease in the absorbance cannot be attributed solely to the conformational changes. The possible O-acetylation of tyrosine residues cannot account for the observed decrease, because the experiments on hydroxylamine treatment of modified ovalbumin indicated that the decrease, if caused by the O-acetylation, can hardly be 3% and not 33%. Moreover, it seems inconceivable that the net increase in negative charge on ovalbumin should have such a pronounced effect on the ultraviolet spectra of the protein (33-35). The reason for the marked decrease in absorbance caused by acetylation is not clear to us.

The excitation and emission spectra of ovalbumin and its various acetylated forms are illustrated in Fig. 5. On acetylation

1 F. Ahmad, unpublished results.
modified ovalbumin, as evident from the value of phan residues do not seem to be exposed even in maximally little exposure of tryptophan residues in the native protein. Progressive modification led to an increase in the exposure of tryptophan residues of ovalbumin. However, all three trypto-

the indole chromophores of ovalbumin, these results suggest very slope for N-acetyltryptophanamide was taken to represent 100% regarded as a measure of the volume of the solvent surrounding exposure of the tryptophan residues. Since the slope may be determined to be 0.20, 0.29, 0.51, and 1.20, respectively. The 98% modified ovalbumins and N-acetyltryptophanamide were unmodified ovalbumins following the method of Steiner et al.

by modifying all the amino groups of ovalbumin. Strikingly, successive increase in acetylation of ovalbumin caused apprecia-

tion, the quantum yield of ovalbumin decreased appreciably without a detectable effect on the emission maxima. Similar decreases in quantum yield without any change in emission maxima were observed for the disruption of native conformation in phosphorylase in the pH range of 5.2 to 6.2 (36). As much as 21% decrease in the relative fluorescence intensity was recorded by modifying all the amino groups of ovalbumin. Strikingly, nearly half the decrease occurred on the modification of only 21% of the ε-amino groups; further modification produced smaller changes in the fluorescence. In order to see whether the perturbation of tryptophan fluorescence spectra by acetylation of ovalbumin is due to the real exposure of chromophore to the solvent, we have measured the effect of a neutral perturbant, namely propylene glycol, on the fluorescence of modified and unmodified ovalbumins following the method of Steiner et al. (18). From Fig. 6, the initial slope, dI/dp for native, 21% and 98% modified ovalbumins and N-acetyltryptophanamide were determined to be 0.20, 0.29, 0.51, and 1.20, respectively. The slope for N-acetyltryptophanamide was taken to represent 100% exposure of the tryptophan residues. Since the slope may be regarded as a measure of the volume of the solvent surrounding the indole chromophores of ovalbumin, these results suggest very little exposure of tryptophan residues in the native protein. Progressive modification led to an increase in the exposure of tryptophan residues of ovalbumin. However, all three trypto-

phan residues do not seem to be exposed even in maximally modified ovalbumin, as evident from the value of dl/dp, which was much lower than the limiting value for N-acetyltryptophanamide.

Hydrodynamic Properties—From Table II it is evident that a successive increase in acetylation of ovalbumin caused apprecia-

ble increases in viscosity; the value for maximally modified (98%) protein was more than twice that for the native protein. Here it would be instructive to compare the hydrodynamic behavior of modified ovalbumins with those listed in Table III for ovalbumin in different denaturing media. The intrinsic viscosity of 98% modified ovalbumin, which is only about 27 to 30% of the values for the linear and cross-linked randomly coiled protein, is also 10% lower than that for the acid-denatured ovalbumin, but it is 20% greater than the viscosity of heat-denatured ovalbumin (37); the latter is comparable to the intrinsic viscosity of 93% acetylated ovalbumin.

The intrinsic viscosity of ovalbumin depends both on its shape, represented by ν, the viscosity increment, as well as on the extent of its hydration, ∑mivi, as evident from the relation (38):

\[ [\eta] = \nu (\bar{\eta} + \sum m_i \bar{v}_i^\rho) \] 

(5)

where \( \bar{v}_i^\rho \) is the partial specific volume of ovalbumin and \( m_i \) is the gram of ith component of the solvent with partial specific volume, \( v_i^\rho \), bound to 1 g of the dry protein. With \( \bar{v}_i = 0.749\text{ ml per g}, m_i = 0.2\text{ g per g of dry protein} \) (38) and \( \bar{v}_i^\rho = 1\text{ ml per g}, the viscosity increment, } \nu, \text{ for native ovalbumin was calculated with Equation 5 and was } 4.1, \text{ which corresponds to an axial ratio, } a/b, \text{ of } 3.5 \text{ and to a frictional ratio, } f/f_0, \text{ of } 1.15.

![Fig. 6. Effect of propylene glycol on the fluorescence intensity of N-acetyltryptophanamide (A); and proteins: native ovalbumin (○), 21% acetylated ovalbumin (■), 98% acetylated ovalbumin (△). Proteins (0.3 mg per ml), N-acetyltryptophanamide (0.003 mg per ml), and propylene glycol (50%, v/v) were prepared separately in phosphate buffer, pH 7.0, ionic strength 0.15. Varying volumes of the propylene glycol solution were added to 1.0 ml of the protein or acetyltryptophanamide and made up to 3 ml with the same buffer. Fluorescence was recorded at 25° with 2-mm slits. The excitation and emission wavelengths were 280 and 355 nm, respectively. The observed fluorescence intensities were corrected for blanks containing the corresponding concentrations of propylene glycol. The corrected values were then used to calculate the fluorescence intensities relative to the fluorescence intensity of the corresponding solution in the absence of propylene glycol.](http://www.jbc.org/)

![Table II](http://www.jbc.org/)

**Table II**

Viscosity data for native and modified ovalbumins at pH 7.0, ionic strength 0.15, and 25°

| ε-NH₂ groups modified | Intrinsic viscosity | Viscosity increment | a/b | f/f₀ | L
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>ml/g</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>3.9</td>
<td>4.1</td>
<td>3.5</td>
<td>1.15</td>
<td>A</td>
</tr>
<tr>
<td>21</td>
<td>4.4</td>
<td>4.6</td>
<td>4.0</td>
<td>1.18</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>4.7</td>
<td>5.0</td>
<td>4.3</td>
<td>1.20</td>
<td>134</td>
</tr>
<tr>
<td>62</td>
<td>5.5</td>
<td>5.6</td>
<td>4.8</td>
<td>1.24</td>
<td>143</td>
</tr>
<tr>
<td>66</td>
<td>5.7</td>
<td>6.0</td>
<td>5.1</td>
<td>1.28</td>
<td>151</td>
</tr>
<tr>
<td>93</td>
<td>6.7</td>
<td>6.9</td>
<td>5.8</td>
<td>1.30</td>
<td>165</td>
</tr>
<tr>
<td>98</td>
<td>8.4</td>
<td>8.1</td>
<td>7.1</td>
<td>1.38</td>
<td>188</td>
</tr>
</tbody>
</table>

* F. Ahmad, unpublished results.

![Table III](http://www.jbc.org/)

**Table III**

Intrinsic viscosities of ovalbumin at 25° under different experimental conditions

<table>
<thead>
<tr>
<th>Experimental condition</th>
<th>Intrinsic viscosity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native, phosphate buffer, pH 7.0, ionic strength 0.15</td>
<td>3.9*</td>
<td>(38)</td>
</tr>
<tr>
<td>pH 1.0, ionic strength 0.15</td>
<td>9.3*</td>
<td>(38)</td>
</tr>
<tr>
<td>Ovalbumin heated and cooled, phosphate buffer, pH 8.0</td>
<td>7.0*</td>
<td>(37)</td>
</tr>
<tr>
<td>Guanidine hydrochloride, 6 M, phosphate buffer, pH 6.8</td>
<td>27.0</td>
<td>(16)</td>
</tr>
<tr>
<td>Guanidine hydrochloride, 6 M, plus 0.1 M β-mercaptoethanol, pH 5.1</td>
<td>31.0</td>
<td>(16)</td>
</tr>
<tr>
<td>93% N-acetylated ovalbumin, phosphate buffer, pH 7.0, ionic strength 0.15</td>
<td>6.5</td>
<td>This study</td>
</tr>
<tr>
<td>98% N-acetylated ovalbumin, phosphate buffer, pH 7.0, ionic strength 0.15</td>
<td>8.4</td>
<td>This study</td>
</tr>
</tbody>
</table>

* F. Ahmad, unpublished results.

* Values calculated from reduced viscosities.
The latter value compares well with 1.17 determined by another procedure (38). The equivalent hydrodynamic radius, \( R_e \), was calculated for the native and 21\% acetylated ovalbumins with appropriate equations and its values were 25.3 and 25.4 Å, respectively. The value of \( R_e \) calculated from the diffusion coefficient data was 27.6 Å (38). Such calculations were not repeated for higher modified proteins because, as judged by \( v \) values, they deviate significantly from globular shape. Therefore, other calculations were performed on microscope slides, containing 4 ml of 1.5\% (w/v) agar prepared in 0.05\% sodium azide. The distance between the trough and the well was 4 mm. Electrophoresis of the antigen, placed in the 1.5\% (w/v) sodium azide. The distance between the trough and the well was 4 mm. Electrophoresis of the antigen, placed in the central wells, was carried out with the sodium barbital buffer, pH 8.6, for 93\% and 98\% modified ovalbumins. All the slides were stained with Amido Schwartz dye. The number refers to the per cent of lysine residues modified in ovalbumin. Unnumbered peripheral wells contained native ovalbumin.

Unnumbered peripheral wells contained native ovalbumin.

\[ L = 5.82 \times 10^{-8} \left( \frac{[\eta]_M}{M} \right)^{1/3} \left( \frac{a}{b} \right)^{1/3} \quad (6) \]

where \( p \) is the axial ratio, \( a/b \), and \( M \) is the molecular weight. The values of \( [\eta] \), \( v \), and \( a/b \) listed in Table II were used in the computation of \( L \) for ovalbumins modified to 28\% or above. The trend of the variations of \( f/f_0 \) and \( L \) suggests that, with the progress in the extent of acetylation, the asymmetry of the ovalbumin molecule increases significantly due to partial denaturation. The gel filtration data given in Table IV for ovalbumin and its six modified forms were used in the estimation of Stokes radius, \( a \). Two calibration curves were obtained according to Equations 2 and 3 with the marker proteins (see Fig. 7). The Stokes radii computed from the curves are given in Table IV; the seventh column shows the average value. The last column gives the frictional ratio, \( f/f_0 \), which was calculated from the Stokes radius by means of the equation:

\[ f/f_0 = \frac{a}{(3\sqrt{2}/4\pi M)^{1/3}} \quad (7) \]

The values of \( f/f_0 \) for modified and unmodified proteins listed in Table IV are invariably somewhat higher than those computed from the viscosity data, but the trend of the variation of \( f/f_0 \) determined by the two methods is the same.

**Immunological Properties—**A single band both in immunodiffusion and immunoelectrophoresis (see Fig. 8) supported our contention that the modification of lysine residues of ovalbumin was uniform. The conformational change in 96\% modified protein does not seem to be enough to give spurs of the type observed for 93\% and 98\% modified ovalbumins.

**TABLE IV**

<table>
<thead>
<tr>
<th>ε-NH₂ groups modified</th>
<th>( V_s )</th>
<th>( K_{av} )</th>
<th>( K_d )</th>
<th>Stokes radius</th>
<th>( f/f_0 )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Curve A</td>
<td>Curve B</td>
<td>Mean</td>
<td></td>
<td></td>
</tr>
<tr>
<td>%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>156</td>
<td>0.511</td>
<td>0.551</td>
<td>A</td>
<td>27.3</td>
</tr>
<tr>
<td>21</td>
<td>140</td>
<td>0.468</td>
<td>0.505</td>
<td>A</td>
<td>29.3</td>
</tr>
<tr>
<td>28</td>
<td>145</td>
<td>0.432</td>
<td>0.466</td>
<td>A</td>
<td>30.2</td>
</tr>
<tr>
<td>62</td>
<td>120</td>
<td>0.396</td>
<td>0.437</td>
<td>A</td>
<td>31.7</td>
</tr>
<tr>
<td>66</td>
<td>136</td>
<td>0.390</td>
<td>0.396</td>
<td>A</td>
<td>33.7</td>
</tr>
<tr>
<td>93</td>
<td>128</td>
<td>0.300</td>
<td>0.334</td>
<td>A</td>
<td>35.8</td>
</tr>
<tr>
<td>98</td>
<td>118</td>
<td>0.237</td>
<td>0.256</td>
<td>A</td>
<td>38.8</td>
</tr>
</tbody>
</table>

**Fig. 7.** Gel filtration data for marker proteins and modified ovalbumins plotted according to Equations 2 and 3 for the estimation of Stokes radii of the modified ovalbumins. The data were obtained by running 5 to 10 mg of protein through a Sephadex G-100 column at a flow rate of 20 ml per hour, collecting fractions of 2-ml size. The chromatographic parameters of the column were: \( V_s \), 224 ml; \( V_i \), 129 ml; and \( V_v \), 85 ml. ( ) marker proteins; (O) ovalbumins modified to varying degrees. The number refers to the per cent of lysine groups modified in ovalbumin. The equations for the two plots are:

\[ A. \quad (-\log K_{av})^{1/2} = 0.2526 a - 0.1903 \]

\[ B. \quad erfc^{-1} K_d = 0.0366 a - 0.5989 \]

**Fig. 8.** Immunodiffusion and immunoelectrophoresis of native and modified ovalbumins. For immunodiffusion (top two), slides were made from 4 ml of 1.5\% (w/v) agar solution in 0.15\% NaCl containing 0.02\% (w/v) sodium azide. The wells were cut at 8-mm distance with a 15-gauge hypodermic needle. Antigen solutions were kept in the peripheral wells and the antiserum was placed in the central wells. The precipitin lines were allowed to develop for about 24 hours. Immunoelectrophoresis was also performed on microscope slides, containing 4 ml of 1.5\% (w/v) agar prepared in 0.05\% sodium barbital buffer, pH 8.6, and 0.02\% (w/v) sodium azide. The distance between the trough and the well was 4 mm. Electrophoresis of the antigen, placed in the wells, was carried out with the sodium barbital buffer, pH 8.6, for 5 hours at 1 mm per gel. After electrophoresis, antiserum was poured in the trough and the precipitin lines allowed to develop for about 24 hours. All the slides were stained with Amido Schwartz dye. The number refers to the per cent of lysine residues modified in ovalbumin. Unnumbered peripheral wells contained native ovalbumin.
The immunological differences among the native and modified ovalbumins have been quantitatively investigated by quantitative precipitin titration (see Fig. 9). Evidently, the extent of cross-reactivity of ovalbumin decreased with chemical modification without changing the equivalence zone of the titration curve. Since the supernatants were not analyzed to determine the presence of soluble antigen-antibody complexes or of the free antigen, a calculation of antigen to antibody ratio in the precipitates and hence of the number of antigenic determinants is not justified. Nevertheless, an interesting feature of the titration data is that even the maximally modified ovalbumin retained 40% precipitability. Considering the formation of soluble antigen-antibody complexes, the actual cross-reactivity of the modified ovalbumin may be even higher.

**DISCUSSION**

Previous studies have shown that acetylation of amino groups in proteins is rarely complete, the extent of modification varies from 60 to 90% (9, 23). Further, acetylation often led to the modification of other functional groups, particularly tyrosine. The procedure used in this study permits rather specific as well as almost complete acetylation of the lysine residues of ovalbumin, presumably because all the ε-amino groups in ovalbumin are accessible to solvent, as evident from the titration data of Cannan et al. (25).

Available data on intrinsic viscosity (see Table III), frictional ratio (38, 39), and second virial coefficient (40) for native ovalbumin are consistent with its compact and globular conformation. That the latter is considerably altered upon acetylation has been unequivocally demonstrated by our results. Thus the absence of linearity in the plot between net charge and electrophoretic mobility, marked changes in the ultraviolet absorption and fluorescence spectra, increase in tryptic digestion, and decrease in immunological activity, all suggested definite conformational changes in acetylated ovalbumins. However, more direct and convincing evidence for the marked structural changes in modified ovalbumins were provided by our hydrodynamic results.

**Extent of Conformational Change**—Our optical results showed only qualitatively that acetylation of amino groups of ovalbumin produces unfolding and exposure of the tyrosine and the tryptophan residues. However, all the indole groups were not exposed, even in the maximally modified protein. Further, the latter retains at least 40% immunological activity, which would imply that even fully modified ovalbumin retains regions of ordered structures whose maintenance does not require the chemical integrity of the ε-amino groups. That the fully acetylated ovalbumin retains considerable residual structure is revealed by the viscosity data of Table III. The hydrodynamic volume of 98% acetylated ovalbumin is approximately comparable to those obtained by acid or heat denaturation, but it is certainly far less than that found in 6 M guanidine hydrochloride, in which solvent ovalbumin behaves as a cross-linked random coil (16).

The ordered protein structure, which is stabilized by noncovalent interactions (e.g., hydrogen bonds and hydrophobic interactions) and destabilized by conformational free energy, is only marginally more stable than the random coil even under native conditions (41). For ovalbumin, the stability contribution of hydrophobic interactions and hydrogen bonds computed by the procedure of Tanford (41, 42) would be, respectively, −390 and −31 kcal per mol; more realistic estimates would yield considerably lower values. The destabilizing contribution of conformational entropy was calculated by the same method (42) and was +413 kcal per mol. Although such a calculation represents an overestimation of the destabilizing contribution, it does indicate that even small destabilizing contributions available by abolishing positive charge on ovalbumin may be sufficient in disrupting protein conformation.

Chemical modification of the lysine residues may destabilize the native protein conformation due to (a) the increase in electrostatic free energy of the protein molecule, (b) the loss of favorable free energy change of solvation of the positively charged amino groups, and (c) steric repulsions involving the entering groups. The increase in electrostatic free energy of the 98% modified protein, corresponding to an increase of 19 net negative charges, was calculated by equations 26 to 30 of Ref. 43 at an ionic strength of 0.15 and was about +8 kcal per mol at 25°. The value for the radius of the protein sphere was taken from Table IV. The removal of positive charge would deprive the ovalbumin molecule of its favorable free energy of solvation. Further, it is possible that the entering acetyl groups are “steric misfits” for the ordered structure, just as proline and hydroxyproline are for the α-helical conformation of proteins (44). The unfolding of the acetylated ovalbumin appears to be the result of one or a combination of all three factors listed above. It is of interest to note that succinylation (4), maleylation (45), and citraconylation (6), which increase the net negative charge and hence the electrostatic free energy of the protein molecule, are generally effective perturbants, whereas guanidination and amidination, which leave the positive charge on protein intact, do not produce detectable unfolding of proteins (2-4). For the same degree of modification, deamination of ovalbumin (46, 47) exerts relatively less influence than acetylation. Therefore, it appears that in addition to the increase in electrostatic free energy and the loss of free energy of solvation, which appear to be the predominant effects, acetylation of the lysine residue introduces a rather bulky group which tends to destabilize the folded structure of ovalbumin by nonbonded repulsion.

**Acknowledgments**—We thank Dr. Christian B. Anfinsen, Laboratory of Chemical Biology, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Maryland, for reading the earlier version of
this paper. We are deeply indebted to Dr. H. Edelhoch, Clinical Endocrinology Branch, National Institutes of Health, Bethesda, Maryland, and to Dr. Rose G. Mage, Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, for extensive critical discussions and suggestions.

REFERENCES

42. Tanford, C. (1962) J. Amer. Chem. Soc. 84, 4240-4247
46. Maurer, P. H., and Heidelberger, M. (1951) J. Amer. Chem. Soc. 73, 2072-2072
47. Maurer, P. H., Heidelberger, M., and Moore, D. H. (1951) J. Amer. Chem. Soc. 73, 2072-2076
Acetylation of amino groups and its effect on the conformation and immunological activity of obalbumin.
A A Ansari, S A Kidwai and A Salahuddin


Access the most updated version of this article at http://www.jbc.org/content/250/5/1625

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/250/5/1625.full.html#ref-list-1