Effect of Succinylation (3-Carboxypropionylation) on the Conformation and Immunological Activity of Ovalbumin

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The ϵ -amino groups of ovalbumin were modified with succinic anhydride; as many as 16 lysine residues were succinylated (3-carboxypropionylated). The five succinylated derivatives thus prepared were homogeneous with respect to the extent of chemical modification as shown by electrophoretic and immunological data. Succinylation of the amino groups altered electrophoretic mobility and isoionic pH of ovalbumin in the expected direction. U.v.-absorption and fluorescence spectra suggested changes in the microenvironment of the chromophores in the modified proteins. The difference-spectral results showed greater exposure of tyrosine and tryptophan residues in the succinylated ovalbumin. Increase in susceptibility to tryptic digestion, Stokes radius and intrinsic viscosity of native ovalbumin, which was observed on successive increase in the chemical modification, demonstrated a conformational change that was proportional to the extent of modification. The loss of immunological reactivity caused by chemical modification also indicated a conformational change in succinylated ovalbumin. The fact that the intrinsic viscosity of maximally modified ovalbumin was less than one-third of that for the completely denatured protein in 6M-guanidinium chloride suggested that the modified protein contained significant residual native structure. The latter presumably accommodates some antigenic determinants accounting for 37% residual immunological activity observed with maximally succinylated ovalbumin.

Chemical modification of proteins with succinic anhydride and other dicarboxylic anhydrides has been successfully used in the study of various aspects of proteins and enzymes. Thus the modification technique was used in protein dissociation (Klotz & Keresztes-Nagy, 1962; Jaenicke *et al.*, 1968), protein hybridization (Penhoet *et al.*, 1967), sequence analysis (DeLange *et al.*, 1969), 'mapping' of the lysine peptides (Butler *et al.*, 1969), introducing the desired functional group (Klotz *et al.*, 1965), and in probing the biological role of amino groups (Prisco *et al.*, 1970). Since at neutral pH a protein acquires a net charge of -2 on succinylation (3-carboxypropionylation) of one amino group, the reaction can profitably be used to investigate the role of charge on the stability of the folded native protein conformation (Shio *et al.*, 1972). Although succinylation of amino groups produced a large conformational change in some proteins (Jonas & Weber, 1970), it had little or no effect on others (Shio *et al.*, 1972). The change in protein conformation, wherever it occurred as a result of succinylation, was invariably attributed to the electrostatic effects (Habeeb *et al.*, 1958; Jonas & Weber, 1970); and the steric effect of

the bulk of the entering $\text{COCH}_2\text{CH}_2\text{CO}_2^-$ function, which may also destabilize native protein conformation through non-bonded repulsion, was ignored.

A proper and meaningful understanding of the structural effects of succinylation of protein requires a detailed and systematic study of the physico-chemical, and if possible biological, properties of the modified protein. Further, it would be interesting to see how these properties change with successive increases in the extent of modification. The present paper describes for the first time, to our knowledge, such a systematic study of ovalbumin and its five succinylated derivatives. The present results unequivocally demonstrate a loss of immunological activity and marked disruption of the native conformation of ovalbumin induced by succinylation of its lysine residues. The increase in electrostatic free energy of native ovalbumin owing to the chemical modification appears to be an important, but not the only, destabilizing factor.

Experimental

Materials

Ovalbumin was either prepared from egg white as described by Kekwick & Cannan (1936) and crystallized five times, or was obtained from Sigma Chemical

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Co. (St. Louis, MO, U.S.A.). Both the preparations were free from extraneous proteins as shown by column chromatography (Sephadex gel and CM-cellulose), polyacrylamide-gel electrophoresis and immunological techniques. The mol.wt. of the protein was taken as 43000 (Castellino & Barker, 1968). Cytochrome *c*, ribonuclease A, α -chymotrypsinogen, trypsin and bovine serum albumin were purchased from Sigma. The materials used in polyacrylamide-gel electrophoresis and gel-filtration experiments were the same as reported previously (Ansari & Salahuddin, 1973a). Succinic anhydride was purchased from Riedel, Seelze, Hannover, Germany. Other reagents were of analytical grade.

Methods

Succinylation of ovalbumin. Succinylated derivatives of ovalbumin were prepared as described by Klotz (1967) in 0.1M-sodium phosphate buffer, pH7.0–7.4. Increasing amounts of solid succinic anhydride (50–1000-fold molar excess with respect to protein) were added to the protein solution (5mg/ml), in small portions, over a period of 1h with gentle stirring, and the pH of the reaction mixture was maintained by addition of 0.2M-NaOH. The solution was left for 30min at room temperature (25°C). It was dialysed first against water and then against sodium phosphate buffer, pH7.0, $I = 0.15M$. The extent of chemical modification of the lysine residues of ovalbumin was determined by ninhydrin reaction as described by Moore & Stein (1954).

Isoionic pH. A mixed-bed ion-exchange resin column was prepared as described by Dintzis (1952) from Dowex 50W-X8 and Dowex 1-X4 resins in NH_4^+ , H^+ , acetate and OH^- ion cycles. Extensively dialysed ovalbumin (or its succinylated form) solution was passed through the column and the pH of the eluate measured immediately on an Elico pH-meter, model LI-10 with glass and calomel electrodes.

Determination of protein. Protein concentration was measured as a routine by the method of Lowry *et al.* (1951). However, for the measurements of viscosity and molar extinction coefficient, the concentration was determined by taking known weights of isoionic protein solution in weighing bottles and heating them at 106°C to constant weights. The molar extinction coefficients of ovalbumin and succinylated ovalbumins were determined by measuring the E_{280} of protein solutions (in sodium phosphate buffer, pH7.5, $I = 0.15M$) of known concentrations.

Polyacrylamide-gel electrophoresis. Electrophoresis of ovalbumin and its chemically modified forms was carried out in 7.5% (w/v) polyacrylamide gel by using a discontinuous buffer system (Cabriel, 1971). The separating and stacking gels were respectively run at pH8.0 and 7.0. The buffer system was diethylbarbituric acid/Tris, pH7.0, $I = 0.02M$. About 100 μg of

protein in 0.1 ml of large-pore gel was applied and a current of 7mA per tube (0.5cm $\times$ 7.0cm) was passed for 1h. The gels were stained in 1% Amido Schwartz solution in 7% (v/v) acetic acid and destained mechanically with water and 7% acetic acid.

Spectral measurements. Light-absorption measurements in the u.v. region were made either on a Carl Zeiss VSU2-P or on a double-beam Beckman model DK-2A ratio recording spectrophotometer. A Carl Zeiss spectrocoulometer (Spekol) was used in the light-absorption measurements in the visible range.

Tryptic digestion. Hydrolysis of modified and unmodified ovalbumins was carried out in 0.1M-sodium phosphate buffer, pH7.5, at 34°C as described by Paik & Kim (1972), with slight modification as reported previously (Ansari *et al.*, 1975).

Gel filtration. The Stokes radii of succinylated ovalbumins were determined by a gel-filtration method. Columns of Sephadex G-100 (1.92cm $\times$ 55.5cm) and G-200 (2.7cm $\times$ 50cm) were prepared in sodium phosphate buffer, pH7.0, $I = 0.15M$, by the standard procedure described elsewhere (Ansari & Salahuddin, 1973a). The two columns were calibrated with marker proteins, which, with their Stokes radii (Andrews, 1970) in parentheses were: cytochrome *c* (1.64nm), ribonuclease A (1.92nm), α -chymotrypsinogen (2.24nm), ovalbumin (2.73nm), bovine serum albumin monomer (3.55nm) and bovine serum albumin dimer (4.30nm). The void volumes of the columns were determined by passing Blue Dextran through them. About 20mg of protein was passed through the column and eluted with the phosphate buffer, pH7.0, $I = 0.15M$, in 3ml fractions with a flow rate of 30ml/h. The elution volume of each protein was determined in duplicate and the values from different runs were normalized in terms of distribution coefficient, K_d , and available distribution coefficient, K_{av} . Least-squares analysis of the data was done by eqns. (1) and (2) put forward by Laurent & Killander (1964) and by Ackers (1967) respectively:

$$(-\log K_{av})^{\pm} = Aa + B \quad (1)$$

$$\operatorname{erfc}^{-1} K_d = a_0 a - b_0 \quad (2)$$

where a is the Stokes radius of the protein (in nm) with distribution coefficient, K_d ; A , B , a_0 and b_0 are calibration constants; and $\operatorname{erfc}^{-1} K_d$ is the inverse error function complement of K_d . For the Sephadex G-100 column, the values of A , B , a_0 and b_0 were respectively 0.2526, -0.256, 0.4170 and -0.3810; the corresponding values for the Sephadex G-200 column were 0.2071, -0.1170, 0.2604 and -0.2751 respectively. The Stokes radius of a given protein computed from the elution volumes determined on the two columns by eqns. (1) or (2) was the same.

Measurement of viscosity. Viscosity was measured as described by Ahmad & Salahuddin (1974), by using a Cannon-Fenske viscometer with a flow time of 416s

for 5ml of water at 25°C. The times of fall, t_0 and t respectively for solvent and protein solution, were measured at $25 \pm 0.1^\circ\text{C}$. The intrinsic viscosity, $[\eta]$, was computed by the method of least squares by using the following relationship (Tanford, 1955):

$$[\eta] = \lim_{c \rightarrow 0} \frac{t - t_0}{t_0 c} + \frac{(1 - \bar{v}_2)\rho_0}{\rho_0} \quad (3)$$

where c is the protein concentration in g/ml and ρ_0 is the density of the solvent which was determined by weighing known volumes of the solvent, i.e., sodium phosphate buffer, pH 7.5, $I = 0.15\text{M}$. The partial specific volume, $\bar{v}_2$, was taken as 0.748 ml/g (Dayhoff *et al.*, 1952) for both ovalbumin and its succinylated derivatives; $\bar{v}_2$ values for native and completely succinylated bovine serum albumin are 0.734 (Dayhoff *et al.*, 1952) and 0.716 ml/g (Habeeb, 1966) respectively. Assuming a similar decrease in $\bar{v}_2$ for succinylated ovalbumin, the increase in intrinsic viscosity, according to eqn. (3), would be less than 0.4%, which is insignificant in comparison with our experimental error of about 2%.

Immunological methods. Antiserum against native ovalbumin was raised in rabbits as described previously (Ansari & Salahuddin, 1973*b*). Double diffusion was performed by the standard procedure (see Kabat & Meyer, 1961). Quantitative precipitin titration of ovalbumin and its modified forms was carried out by the method of Heidelberger & Kendall (1935) as modified in this laboratory (Ansari & Salahuddin, 1973*b*).

Results

Reaction of succinic anhydride with ovalbumin

Succinic anhydride reacts primarily with amino groups (α - and ϵ -amino) of proteins (Habeeb *et al.*, 1958). At neutral pH (~ 7.4), the α -amino group of ovalbumin is expected to react with the anhydride more readily than the ϵ -amino groups. The reagent may also react with the tyrosine residues. However, the N -terminal glycine residue of ovalbumin is acetylated (Narita, 1961), and, under the conditions of our experiments, the O -succinylated tyrosine derivative would be highly unstable owing to spontaneous ester hydrolysis catalysed by the intramolecular free carbonyl groups (Gounaris & Perlman, 1967). Further, when succinylated ovalbumins were treated with 1 M-hydroxylamine at pH 7.5 (Riordan & Vallee, 1967), and the E_{278} was recorded against the blank containing equal concentration of hydroxylamine, no significant increase in absorbance was noticed, precluding the acylation of the tyrosine residues. In view of these considerations it is inferred that succinylation must have occurred primarily at the lysine residues of ovalbumin. As shown in Fig. 1, the extent of modification

was higher at higher succinic anhydride concentrations. As many as 82%, or about 16 lysine residues of ovalbumin were modified at a molar ratio of the anhydride to the ϵ -amino group of 53:1. It is noteworthy that, out of the 16 modified lysine residues, only six were succinylated readily and the remaining ten reacted gradually. Three lysine residues of ovalbumin were inaccessible to succinic anhydride even at a molar ratio of the anhydride to the protein of 1000:1.

Isoionic pH

The isoionic pH values of native and succinylated ovalbumins were measured at a protein concentration of 10 mg/ml or less and are listed in Table 1. At lower degrees of modification, isoionic pH decreased with increase in the extent of modification. Surprisingly, the isoionic pH remained unaltered, when the extent of modification increased from 66 to 82%.

Polyacrylamide-gel electrophoresis

Results of polyacrylamide-gel electrophoresis of native and modified ovalbumins are shown in Plate 1. The number of protein bands in each case was the same. There seems to be a correlation between the relative mobility towards the anode, i.e. R_m value, and the extent of modification up to 57% succinylation, beyond which the increase in extent of modification from 66 to 82% had no detectable effect on the R_m value (see Table 1).

Spectral properties

The u.v.-absorption spectra of ovalbumin and its succinylated derivatives in phosphate buffer, pH 7.0, $I = 0.15\text{M}$, are depicted in Fig. 2. Native ovalbumin absorbs maximally at 279 nm and the absorption decreases considerably on succinylation of the lysine residues of the protein (see Table 1). The decrease in molar extinction was roughly $600\text{cm}^2 \cdot \text{mol}^{-1}$ for the modification of one lysine residue. A similar, although somewhat smaller, decrease in extinction was also noted previously during the succinylation of aldolase (Hass, 1964) and pepsinogen (Gounaris & Perlman, 1967).

The difference spectra of 57%-, 66%-, and 82%-succinylated ovalbumins against native ovalbumin are shown in Fig. 3. The notable features of the spectra of 66%-, and 82%-succinylated ovalbumins are a shoulder at 272 nm and three peaks near 282, 286 and 294 nm respectively. These fine structures are mostly retained in the spectrum of 57%-succinylated ovalbumin with the difference that the peak near 286 nm appears as a shoulder (see Fig. 3). Similar spectral features were also noted for acid-denatured ovalbumin (Yanari & Bovey, 1960).

Fig. 1. Determination of ninhydrin colour of succinylated ovalbumins

Increasing amounts of the protein in 1 ml were incubated with 1 ml of freshly prepared ninhydrin reagent in boiling water for 15 min. The mixture was then diluted with 50% (v/v) ethanol and the E_{570} recorded. Native ovalbumin (—); ovalbumin treated with increasing molar excess of succinic anhydride: 50 (----), 100 (-.-), 200 (-.-.-), 500 (-.-.-.-) and 1000 (.....). The values of m and m' , representing the slopes of the straight lines for unmodified and modified ovalbumins respectively, were determined by the method of least squares. The percentage modification, $100(1-m'/m)$, was then calculated and plotted against the increasing concentration of succinic anhydride as shown in the inset.

Table 1. Molecular properties of succinylated ovalbumins

The relative mobility was calculated by dividing the observed mobility by that of Bromophenol Blue; ν , viscosity increment; a/b , axial ratio; f/f_0 , frictional ratio; L , length of the equivalent ellipsoid.

Molar ratio: anhydride/protein	ϵ -Amino groups modified (%)	Isoionic pH	Relative mobility	$10^{-4} \times$ Molar extinction at 280 nm	$[\eta]$	ν	a/b	f/f_0	L (nm)
0	0	4.9	0.28	3.01	3.9	4.1	3.5	1.17	—
50	31	4.4	0.36	2.70	—	—	—	—	—
100	50	4.3	0.38	2.43	4.7	5.0	4.3	1.20	—
200	57	4.3	0.42	2.38	—	—	—	—	—
500	66	4.1	0.46	2.14	5.7	6.0	5.1	1.26	15.1
1000	82	4.1	0.46	2.04	7.6	8.0	6.5	1.35	17.6

The excitation and emission spectra of native and succinylated ovalbumins are depicted in Fig. 4. The corrected excitation and emission maxima of the

modified and unmodified proteins were found to be the same and were 280 and 335 nm respectively. Succinylation of the lysine residues of ovalbumin

— 0 31 50 57 66 82

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EXPLANATION OF PLATE I

Polyacrylamide-gel electrophoresis of ovalbumin and succinylated ovalbumins at pH7.0

The number refers to the percentage modification of lysine residues in ovalbumin. In the diagram, the band for 82%-modified ovalbumin appears to be faster moving than that for 66% modified ovalbumin, but this is because of a difference in the distance moved by Bromophenol Blue in the two gels; the R_m values for 82% and 66% modified ovalbumins were actually the same (see Table 1).

EXPLANATION OF PLATE 2

Immunodiffusion of modified and unmodified ovalbumins

Rabbit antiserum against ovalbumin was placed in the central well; succinylated ovalbumins in optimal concentration were taken in the peripheral wells. Precipitin lines were allowed to develop at 4°C for 2 days and stained with acid Fuchsin. The numbers represent the percentage succinylation of ovalbumin and OV refers to the unmodified ovalbumin.

Fig. 2. U.v.-absorption spectra of native and succinylated ovalbumins

The protein solution (1.5mg/ml) was prepared gravimetrically in phosphate buffer, pH7.0, $I = 0.15M$. Native ovalbumin (-----); modified ovalbumins: 31% (.....), 50% (-.-.-), 57% (-.-.-), 66% (-.-) and 82% (—).

produced an appreciable decrease in the relative fluorescence intensity. The fluorescence intensity was diminished by 40% for 82%-succinylated ovalbumin. To see whether the observed fluorescence quenching is due to the entering carboxylate group, we have determined the effect of 0.03M-sodium succinate on the fluorescence intensity of native ovalbumin at 335nm in phosphate buffer, pH7.0, $I = 0.15M$. Strikingly, an appreciable (17%) decrease in the fluorescence intensity of the native ovalbumin was observed in presence of 0.03M-sodium succinate.

Tryptic hydrolysis

Tryptic digestion of native and two succinylated ovalbumins (66 and 82%) was measured in 0.1M-phosphate buffer, pH7.5, as a function of time and

Fig. 3. Difference spectra of succinylated ovalbumins in phosphate buffer pH7.0, $I = 0.15M$

Protein solutions (5mg/ml) were prepared from isoionic preparations. The native and modified ovalbumins were taken respectively in the reference beam and sample beam. Modified ovalbumins: 57% (—), 66% (---) and 82% (-.-.-). ΔE represents the difference in specific extinction coefficient.

the results are shown in Fig. 5. Progressive hydrolysis of the protein peptide bond would yield increasing numbers of newly formed α -amino groups which will be available for reaction with the ninhydrin reagent. Thus ΔE_{570} in Fig. 5 would measure the hydrolysis of those peptide bonds in protein that are available for tryptic attack. As judged by ΔE_{570} values, the native ovalbumin was resistant to tryptic attack, an observation that is in agreement with our earlier conclusion (Ansari *et al.*, 1975). The extent of tryptic hydrolysis was appreciable for 66%- and 82%-succinylated ovalbumins.

Gel-filtration results

The elution volume of native ovalbumin from a Sephadex column decreased with successive increase in the extent of succinylation. A decrease of 26ml was observed when 16 lysine residues of ovalbumin were modified (see Table 2). The marked decrease in the elution volume should be attributed to the increase in hydrodynamic volume of the modified proteins. Sephadex columns possess a small but detectable cation-exchange property, especially at lower ionic strengths ($<0.01M$), owing to the presence of a small number of carboxyl groups on the gel. This would lower somewhat the elution volume of a protein such as succinylated ovalbumins with large numbers of net negative charges. However, the ion-exchange effect will be altogether eliminated at the ionic strength of 0.15M used in the present study.

Fig. 4. Excitation and emission spectra of ovalbumin and succinylated ovalbumins

The spectra were recorded at 32°C by using a 1 mm slit. The excitation (left, emission wavelength 335 nm) and the emission spectra (right, excitation wavelength 280 nm) of the protein solutions (1.5 mg/ml) in phosphate buffer, pH 7.0, $I = 0.15M$, were obtained under identical conditions. Native ovalbumin (—), succinylated ovalbumins: 57% (----), 66% (-.-) and 82% (....).

Table 2 shows that the treatments of the gel-filtration data by the procedures of both Laurent & Killander (1964) and Ackers (1967) gave identical values for the Stokes radius within experimental error. The Stokes radius of native ovalbumin increased from 2.7 to 3.5 nm on succinylation of its 16 lysine residues. The diffusion coefficient, D , and the frictional ratio, f/f_0 , for native and modified proteins were calculated from the Stokes radius a , by using the following relationship:

$$D = kT/6\pi\eta a \quad (4)$$

$$f/f_0 = a/(3\bar{v}_2 M/4\pi N)^{\frac{1}{3}} \quad (5)$$

where k is Boltzmann constant ($1.380 \times 10^{-23} J/^\circ C$), T is absolute temperature, M is molecular weight of the protein and η is the viscosity of the solvent. The partial specific volume, $\bar{v}_2$, was taken to be 0.748 (Dayhoff *et al.*, 1952) regardless of the extent of succinylation and concurrent conformational change.

The viscosity of the eluent (sodium phosphate buffer, pH 7.0, $I = 0.15M$) was taken to be $0.00100 Pa \cdot s$. The diffusion coefficient decreased and f/f_0 increased with increase in the degree of succinylation of the lysine residues of ovalbumin.

Intrinsic-viscosity data

The viscosity data of modified ovalbumins are plotted in Fig. 6. The intrinsic viscosity of ovalbumin in phosphate buffer, pH 7.5, $I = 0.15M$, was measured to be 3.9 ml/g at 25°C (see Table 1). Intrinsic viscosity of the protein was enhanced by succinylation of its lysine residues and increased almost twofold when 82% of the lysine residues were modified. The viscosity increment, ν , which is a factor that depends on the shape of the protein molecules was calculated from intrinsic viscosity by eqn. (6):

$$[\eta] = \nu(\bar{v}_2 + \sum m_i \bar{v}_i) \quad (6)$$

where m_i is the amount (g) of the i th component of the solvent system with partial specific volume $\bar{v}_i$, that was bound to 1 g of dry protein. Assuming that water is the only component of the phosphate buffer whose binding affects the hydrodynamic volume of the protein significantly, we may equate $\Sigma m_i \bar{v}_i$ with the amount of water (in g) bound to 1 g of protein, because $\bar{v}_i$ for water is unity. The hydration of ovalbumin has been measured by isopiestic (Bull & Breese, 1968) and nuclear-magnetic-resonance (Kuntz, 1971) methods and the respective values are 0.30 and 0.33 g/g

Fig. 5. Time-course of the tryptic hydrolysis of native and succinylated ovalbumins

To 0.75 mg of protein in 0.1 M-sodium phosphate buffer, pH 7.5, was added an equal amount of trypsin in the same buffer in a total volume of 3 ml, and the mixture incubated at 34°C for different time-intervals. The reaction was stopped by transferring 0.5 ml of the reaction mixture at different time-intervals into 0.5 ml of precooled 1 M-sodium acetate buffer, pH 5.1. Ninhydrin reagent (0.5 ml) was added and the colour developed as described in Fig. 1. The E_{570} was measured and corrected for the two blanks prepared with only trypsin or the protein substrate; ΔE_{570} is the increase in absorbance over the blank values. Native ovalbumin ($\square$), 66%- ($\circ$) and 82%- ($\bullet$) succinylated ovalbumins.

of protein. However, a value of 0.15 g/g is consistent with the hydrodynamic behaviour of ovalbumin (see Kauzmann, 1974). We have used a value of 0.2 g/g of dry ovalbumin, which is similar to that measured by Wang (1954) from the rate of self-diffusion of water in ovalbumin solutions. The value of $\bar{v}_2$ was taken to be 0.748 (Dayhoff *et al.*, 1952). The values of ν for native and modified ovalbumins were thus calculated by eqn. (6) and are given in Table 1. The corresponding values of the axial ratio a/b and frictional ratio f/f_0 were also computed by standard procedures and are listed in Table 1. Since the intrinsic viscosities of 66%- and 82%-succinylated ovalbumins were far greater than that (3–4 ml/g) of a typical globular protein (Tanford, 1961a), the length of the equivalent ellipsoid, L , was calculated by using the following relationship (Yang, 1961);

$$L = 6.82 \times 10^{-8} ([\eta]M)^{\frac{1}{2}} (p^2/\nu)^{\frac{1}{2}} \quad (7)$$

Fig. 6. Intrinsic viscosities of 50%- ($\square$), 66%- ($\bullet$) and 82%- ($\circ$) succinylated ovalbumins in phosphate buffer, pH 7.5, $I = 0.15$ M, at 25°C

The straight lines were drawn by the method of least squares.

Table 2. Gel-filtration data for native and succinylated ovalbumins at 30°C

Elution volumes of 31%- and 50%-succinylated ovalbumins were obtained from a Sephadex G-100 column; other data were obtained on a Sephadex G-200 column. For details, see the text.

Modification (%)	Elution volume (ml)	$(-\log K_{av})^{\dagger}$	$\text{erfc}^{-1} K_d$	Stokes radius (nm)			$10^7 \times D$ (cm ² /s)	f/f_0
				From eqn. (1)	From eqn. (2)	Mean		
0	206.5	0.5009	0.4760	—	—	2.7	8.1	1.17
31	78.0	0.8239	0.8676	3.00	2.99	3.0	7.4	1.28
50	75.5	0.8551	0.9164	3.11	3.11	3.1	7.1	1.33
57	191.0	0.5685	0.5587	3.31	3.20	3.3	6.7	1.41
66	187.0	0.5864	0.5842	3.40	3.30	3.4	6.5	1.46
82	181.0	0.6139	0.6223	3.53	3.45	3.5	6.3	1.50

Fig. 7. Effect of succinylation on the immunological activity of ovalbumin

----, Variation of immunological activity of ovalbumin with increase in its Stokes radius caused by chemical modification; —, Variation of immunological activity of ovalbumin with chemical modification. To 1 ml of rabbit antiserum were added increasing amounts of ovalbumin (or its modified form) and the mixture was incubated first at 45°C for 1 h (Ansari & Salahuddin, 1973a) and then at 4°C for 20 h. The precipitates were washed with cold 0.9% NaCl and dissolved in 5 ml of KCl/HCl mixture pH 2.2, $I = 0.06M$ (Ansari & Salahuddin, 1973b). The E_{280} of the solution was measured and was taken to be a measure of the protein concentration in the precipitate. The precipitation observed with native ovalbumin at the equivalence point was taken to represent 100% precipitation. The percentage of precipitation with succinylated ovalbumins was calculated at the point of maximum precipitation in the precipitin-titration curve.

where p is the axial ratio. The values of L are given in Table 1.

Immunological properties

The results of immunodiffusion of ovalbumin and its five succinylated derivatives are depicted in Plate 2. No spur or intersection was seen with native and

31%, 50% and 57%-modified ovalbumins. However, the precipitin bands for 66%- and 82%-succinylated ovalbumins did not fuse with the band of native ovalbumin, and distinct spurs were seen.

Results on quantitative precipitin titration of native and succinylated ovalbumins against rabbit anti-ovalbumin are shown in Fig. 7, which shows the effect of chemical modification on the extent of immune precipitation. The curve shown in Fig. 7 delineates the variation of immunological activity of ovalbumin with increase in its hydrodynamic volume as measured by Stokes radius. It is clear that modification of as many as 31% lysine residues of ovalbumin (six residues) had very little effect on the antigenic properties of the protein. The decrease in immune precipitation was substantial for 57%-modified protein. Strikingly, the immunological activity of 66%-succinylated ovalbumin was identical, within experimental error, with that measured for the 82%-succinylated derivative.

Discussion

The present results suggest specific modification of lysine amino groups of ovalbumin during its reaction with succinic anhydride. Although all 19 ϵ -amino groups of ovalbumin (Fothergill & Fothergill, 1970) are accessible to H^+ (Cannan *et al.*, 1941) and hence to the solvent, only about 16 were succinylated. As judged by polyacrylamide-gel electrophoresis, immunodiffusion and immunoelectrophoresis, the five succinylated derivatives of ovalbumin were homogeneous with respect to the extent of modification. The decrease in isoelectric pH and increase in anodic mobility in polyacrylamide gel which were noticed with successive increase in the extent of succinylation up to 66% modification are quite expected. However, above 66% modification the changes in these properties did not correlate with increase in modification, possibly owing to a conformational change.

Tyrosine residues of ovalbumin are not accessible in the native conformation (Cannan *et al.*, 1941; Crammer & Neuberger, 1943) and were probably not modified, as described in the Results section. The reason for the marked decrease in E_{280} which occurred on succinylation of ovalbumin is not immediately apparent. Of course, part of the decrease can be ascribed to the disruption of native protein conformation. The difference-spectral results showing marked perturbation of the spectra of phenylalanine (peak at 272 nm), tyrosine (peak near 282 nm) and tryptophan (peaks near 286 and 294 nm) residues in the modified proteins are reminiscent of the spectral features observed by Yanari & Bovey (1960) for acid-denatured ovalbumin.

The tryptophan fluorescence in ovalbumin is considerably quenched in the native state (Teale, 1960)

and was further quenched to a marked extent on succinylation. Thus the fluorescence results taken separately would apparently suggest that the micro-environment of tryptophan residues in the succinylated protein is more compact than that in the native molecule. However, the fact that 0.03M-sodium succinate produced a 17% decrease in the fluorescence intensity of native ovalbumin indicates that the observed quenching in succinylated ovalbumin may be due to the interaction of the protein chromophore with the $\text{COCH}_2\text{CH}_2\text{CO}_2^-$ function. It is noteworthy that Fasman *et al.* (1964) found a large quenching of the fluorescence intensity of the randomly coiled copolymer of DL-glutamic acid and L-tyrosine by carboxyl ions, which they interpreted to be due to carboxyl ion-tyrosine interaction unrelated to hydrogen-bonded carboxylate.

Since succinylation of lysine residues of ovalbumin would make the lysine peptide bonds refractory to tryptic attack, one should expect a decreasing extent of susceptibility to tryptic digestion of the protein with an increase in the extent of modification. By contrast, the extent of tryptic hydrolysis was higher for 82%-succinylated ovalbumin than for 66%-modified protein. This unequivocally demonstrated unfolding of the protein as a result of succinylation.

The changes in Stokes radius and in other related parameters such as frictional ratio and diffusion coefficient of native ovalbumin, which were seen to increase in succinylation, suggested significant expansion of the modified ovalbumins. The increase in hydrodynamic volume of the native protein owing to lysine modification was also demonstrated by the viscosity data, where a nearly twofold increase in intrinsic viscosity was noticed when 16 lysine residues of ovalbumin were succinylated. The intrinsic viscosity of 82%-succinylated ovalbumin is comparable with the value (7.0 ml/g) measured for the heat-denatured protein (Bull, 1940), but it is much lower than the 27 ml/g value found for ovalbumin in 6M-guanidinium chloride (Ahmad & Salahuddin, 1974), where the protein is expected to be in the random-coil conformation (Tanford, 1968). Thus, although succinylated ovalbumin, like the heat-denatured form, is considerably unfolded, it contains substantial native structure which can be removed by 6M-guanidinium chloride.

The disruption of native conformation of ovalbumin produced by an increasing extent of succinylation accounts for the marked decrease in its immunological reactivity. The fact that even the maximally modified ovalbumin retained 37% immunological activity is significant and supports the above conclusion that the modified protein contains regions of native residual structure, which accommodate some antigenic determinants. These structural domain(s) seem to be too tightly folded to be disrupted by succinylation.

Since at neutral pH in water, succinylation of the α -amino groups of ovalbumin appreciably increases the net negative charge on the protein molecule, it would be logical to explain the conformational change in modified proteins in terms of an increase in the electrostatic free energy of the protein molecule. In fact, molecular expansion of bovine serum albumin produced by chemical modification with succinic anhydride (Habeeb *et al.*, 1958; Jonas & Weber, 1970) has been attributed essentially to the electrostatic repulsion between negative charges. This view was tested in the case of succinylated ovalbumin by actual calculation of the electrostatic free energy, W_{el} , by using the following relationship (Tanford, 1961b):

$$W_{el} = \frac{Z^2 e^2}{2\epsilon a} - \left(1 - \frac{\kappa a}{1 + \kappa R}\right) \quad (7)$$

where Z is the net negative charge on ovalbumin of radius a (2.7 nm), R is $a + 0.25$ nm, e is the electronic charge, ϵ is dielectric constant of water and κ is the well-known Debye-Hückel parameter. The net charge on the ovalbumin molecule at pH 7.0 is near -12 (Cannan *et al.*, 1941), which will be increased to -44 in 82%-succinylated protein. The corresponding increase in electrostatic free energy of the protein molecule was calculated by eqn. (7) at 25°C, $I = 0.15$ M, to be 163000 J/mol. It is reasonable to believe that the destabilizing influence of the increase in electrostatic free energy was large enough to overcome the non-covalent interactions, e.g. hydrogen bond, hydrophobic interaction etc., that are responsible for the stability of native ovalbumin conformation, especially when the latter is more stable than the random-coil conformation in concentrated guanidinium chloride solution only by 25000 J/mol at 25°C, as deduced from the thermodynamic studies of the guanidinium chloride denaturation of ovalbumin (Ahmad, 1974). However, an increase in electrostatic free energy of the ovalbumin molecule alone cannot account for the observed conformational change. First, an increase in pH from 7.0 to 12.0 would raise the electrostatic free energy of ovalbumin by 155000 J/mol according to eqn. (7), but this does not produce any detectable conformational change as shown by titration data (Cannan *et al.*, 1941; Crammer & Neuberger, 1943). Second, 50% succinylation and 98% acetylation (Ansari *et al.*, 1975), although producing identical increases in electrostatic free energy, do not influence the native ovalbumin conformation equally, the latter treatment being more drastic than the former. Third, the intrinsic viscosity of 82%-succinylated ovalbumin is about 14% higher than that found for 93%-acetylated ovalbumin (Ansari *et al.*, 1975). Finally, at comparable extents of modification, deamination (Maurer & Heidelberger, 1951; Maurer *et al.*, 1951) was less effective than acetylation (Ansari *et al.*, 1975) in

bringing about conformational change in ovalbumin. It thus seems that besides long-range electrostatic repulsion, the number and bulk of the entering acyl groups also contribute to the instability of the native conformation of ovalbumin through non-bonded repulsion.

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