Exposure of Tyrosine Residues in Proteins. III. The Reaction of Cyanuric Fluoride and *N*-Acetylimidazole with Ovalbumin, Chymotrypsinogen, and Trypsinogen*

Marina J. Gorbunoff

With the Technical Assistance of P. Ettinger

ABSTRACT: The state of tyrosine residues (accessible or buried) in ovalbumin, chymotrypsinogen, and trypsinogen has been examined with cyanuric fluoride and *N*-acetylimidazole. Ovalbumin was found to contain five reactive and four unreactive residues. The reactive ones can be subdivided into four classes of reactivity. There are eight *N*-acetylimidazole-reactive groups.

Chymotrypsinogen contains two reactive and two unreactive residues; reactive residues are of two types. There are three *N*-acetylimidazole-reactive residues. Trypsinogen contains seven reactive and three unreactive residues. The reactive ones can be subdivided into five classes of 2 + 1 + 1 + 1 + 2 residues according to diminishing reactivity. There are five *N*-acetylimidazole-reactive residues.

In the three-dimensional structure of proteins, individual amino acid residues may be present either on the surface of the molecule and accessible to the solvent medium, or buried in the internal hydrophobic regions. As a result of their location within the three-dimensional structure of a protein, groups are characterized by varying degrees of reactivity toward specific reagents. Groups on the surface resemble simple peptides in their reactivity while those buried in the interior have reactivities lower than those of simple peptides and often are not reactive at all. Groups in intermediate classes display a wide spectrum of reactivity. Since the extent of exposure of a residue and its chemical reactivity are related (Timasheff and Gorbunoff, 1967), it becomes possible to probe the environment of such groups by studying their reactivity with specific reagents. Tyrosine residues of proteins can be modified chemically with several reagents. These include cyanuric fluoride (Kurihara *et al.*, 1963), *N*-acetylimidazole (Riordan *et al.*, 1965), and tetranitromethane (Sokolovsky *et al.*, 1966). Studies with the use of these reagents (Timasheff and Gorbunoff, 1967; Gorbunoff, 1967, 1968) have demonstrated the complexity of the problem. It appears, however, that on the basis of comparative studies with several modifying reagents, it will be possible to arrive at definite conclusions concerning the environment of individual tyrosine residues in proteins.

In previous studies, the reactivity with CyF of tyrosine residues in ribonuclease, α-lactalbumin, β-lactoglobulin (Gorbunoff, 1967), pepsinogen, soybean trypsin inhibitor, and ovomucoid (Gorbunoff, 1968) has been reported. The states of the tyrosine residues in ovalbumin, chymotrypsinogen, and trypsinogen have been examined with cyanuric fluoride and *N*-acetylimidazole as probing reagents and compared with their titration properties; circular dichroism studies have been carried out on acetylated ovalbumin, chymotrypsinogen, and trypsinogen; the results of this study are presented in this paper.

Experimental Procedure

Materials. Cyanuric fluoride, purchased from Miles Laboratories, Inc., and Eastman Inorganic Chemical Corp., was distilled before use. Dioxane, Best Grade from Fisher Scientific Co., was distilled twice over potassium hydroxide pellets and stored in a frozen state. *N*-Acetylimidazole, purchased from Eastman Inorganic Chemical Corp., was recrystallized from dry benzene and kept dry over calcium hydride. Ovalbumin, five-times crystalline (lot R2223), was purchased from Mann Research Laboratories, Inc.; chymotrypsinogen, three-times crystalline (lot CG7FB), and trypsinogen, crystalline (lots TG7JH and TG8CT), were purchased from Worthington Biochemical Corp. Ovalbumin and chymotrypsinogen were used without further purification. Trypsinogen was chromatographed on G-75 Sephadex in 0.1 N pH 6 phosphate buffer in the presence of soybean trypsin inhibitor (one part to 50 parts of trypsinogen by weight) to eliminate residual trypsin activity. Only the middle fractions of the peak were taken, dialyzed against 0.1 N acetic acid, and lyophilized.

Methods. Spectroscopic measurements were made at room temperature on a Cary Model 14 recording spectrophotometer. The pH measurements were made at room temperature with a Radiometer 28 pH meter. Protein concentrations were determined spectroscopically in solvents containing 10% dioxane. For ovalbumin, 0.1 M phosphate buffer (pH 7) was used; for chymotrypsinogen and trypsinogen, 0.001 N HCl was used. For the calculation of concentration from the optical density, the values of absorptivity used were 7.12 dl/cm g at 280 mμ for ovalbumin (McKenzie *et al.*, 1963), 20.0 dl/cm g at 282 mμ for chymotrypsinogen (Wilcox *et al.*, 1957), and 13.9 dl/cm g at 280 mμ (Pechère and Neurath, 1957) for trypsinogen. The molecular weight of ovalbumin was taken as

* Publication No. 638 from the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154. *Received January 6, 1969.* This work was supported in part by the National Institutes of Health Grant No. GM 14603.

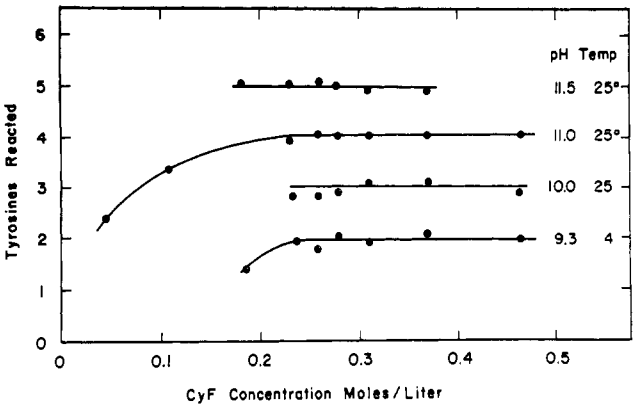

FIGURE 1: Dependence of ovalbumin tyrosine reactivity upon cyanuric fluoride concentration at 4 and 25°. Ovalbumin concentration was 6 mg/ml.

45,000 (Lewis *et al.*, 1950), that of chymotrypsinogen as 25,000 (Wilcox *et al.*, 1957), and that of trypsinogen as 24,500 (Kay *et al.*, 1961).

Spectrophotometric titrations were carried out in 1 M $KHCO_3$ buffer containing 10% dioxane by the difference spectral technique (Wetlaufer, 1962). The ultraviolet spectra were recorded against protein solutions dissolved in 0.1 M phosphate buffer (pH 7.0) in the case of ovalbumin and in 0.001 N HCl in the case of chymotrypsinogen and trypsinogen immediately after mixing and at 10-min periods thereafter. The optical density within 3 min after mixing was taken as the final value for ovalbumin at pH values above 13 (Crammer and Neuberger, 1943). For chymotrypsinogen and trypsinogen, optical densities after 15 min were taken as the final values at pH 13.5. The maxima of the difference spectra were found to be at 297 mμ for ovalbumin and at 296 mμ for chymotrypsinogen and trypsinogen. The number of ionized tyrosine residues was calculated for ovalbumin and trypsinogen using an extinction coefficient of 2300 for the phenoxide ion (Crammer and Neuberger, 1943; Sage and Singer, 1962); for chymotrypsinogen, the corresponding extinction coefficient was taken as 2400 (Marini and Wunsch, 1963).

Reaction with Cyanuric Fluoride. The procedure used was the same as previously described (Gorbunoff, 1967, 1968). After completion of cyanuration, the pH of the reaction mixture was adjusted to 13.2 in the case of ovalbumin and to 13.4–13.5 in the case of chymotrypsinogen and trypsinogen. The ultraviolet spectrum was recorded between 290 and 340 mμ within 5 min for ovalbumin, 10 min for chymotrypsinogen, and 15 min for trypsinogen. The ultraviolet spectrum was recorded against a standard of the same concentration in a solvent containing 10% dioxane. For ovalbumin, 0.1 M phosphate buffer (pH 7.0) was used, while for chymotrypsinogen and trypsinogen, 0.001 N HCl was used. All three proteins were used at concentrations of 6 mg/ml; the ultraviolet spectra were recorded in a 2-cm path-length cell.

It was found that in the case of all three proteins, there was no difference in ultraviolet absorption in the 290–340-mμ region between untreated protein dissolved in a solvent containing 10% dioxane (phosphate buffer pH 7 or 0.001 N HCl) and the cyanuric fluoride treated protein adjusted to pH 7.

The number of moles of tyrosine residues which had reacted under any given conditions of pH, cyanuric fluoride concentrations, and temperature was calculated in the same manner as the spectrophotometric titrations; the optical density at the wavelength of maximal absorption (297, 296, and 296 mμ) was divided by the product of the molar concentration of the protein with the molar extinction coefficient of phenoxide ion in the given protein. This gives the number of moles of unreacted tyrosine residues.

Method of Data Analysis. The tyrosine residues in a protein were classified on the basis of changes in their reactivity with cyanuric fluoride caused by changes in pH, temperature, or both. This was done as follows. In preliminary experiments, the number of reactive tyrosine residues in a protein was determined at 25 and 3° as a function of pH. The protein and cyanuric fluoride concentrations were kept constant. This results in a pH profile for a given protein. On the basis of this profile, pH values close to whole numbers of reacting tyrosine were selected and concentration curves (experiments with increasing concentrations of cyanuric fluoride) were obtained at these pH values. According to criteria established by Kurihara *et al.* (1963), a plateau in a concentration curve indicates that a limit has been reached with respect to tyrosine reactivity. Each plateau is taken to mean a discrete state in tyrosine residue reactivity and the number of such plateaus gives the number of different types of tyrosine residues in any given protein (Kurihara *et al.*, 1963).

Acetylation. Acetylation with *N*-acetylimidazole was carried out according to the method of Riordan *et al.* (1965), except that for chymotrypsinogen and trypsinogen chromatography on a Sephadex G-25 column was replaced by dialysis. Acetylation of trypsinogen was carried out in the presence of 2 weight % of soybean trypsin inhibitor to inhibit residual trypsin activity.

Circular Dichroism. Circular dichroism experiments were carried out on a Cary Model 60 spectropolarimeter equipped with a Model 6001 circular dichroism accessory. The spectra were measured between 330 and 250 mμ in a 1.0-cm cell and between 270 and 185 mμ in a 0.01-cm cell. The results were calculated as the residue molar ellipticity, $[\theta]$, without correction for the refractive index of the solvent, and are reported in these units.

The instrument employed was calibrated with camphorsulfonic acid. Its stability and the reproducibility of results were regularly checked by experiments with standard substances, such as known proteins and polypeptides, as well as by repeating runs on the research samples of interest.

Results

Ovalbumin. Ovalbumin contains nine tyrosine residues (Tristram, 1953), of which only about two titrate in the pH range of 9.5–12 (Crammer and Neuberger, 1943); there is time-dependent ionization above pH 12.3 (Tramer and Shugar, 1959); and at pH 13, the ionization is instantaneous (Crammer and Neuberger, 1943). The titration curve of ovalbumin in 1 M $KHCO_3$ buffer containing 10% dioxane showed no significant differences from that reported by Crammer and Neuberger (1943) in the pH range 8.3–11.8; however, time-dependent ionization manifests itself already at pH 11.5. Above pH 11.8, 10% dioxane destabilizes the structure to such an extent that the titration curve is displaced by about 1 pH unit.

Preliminary experiments were carried out with 0.232 M

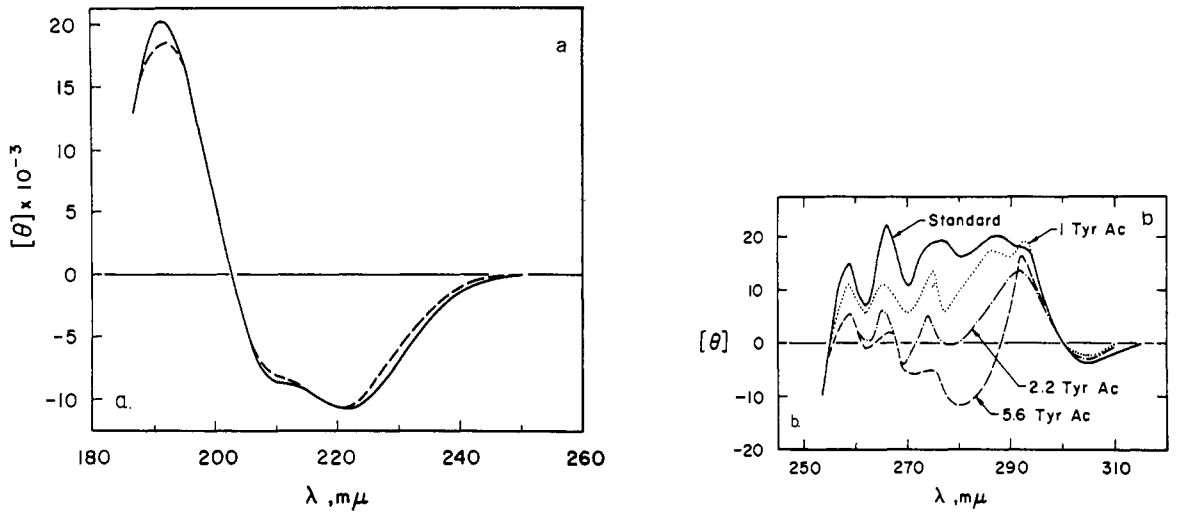

FIGURE 2: Circular dichroism spectra of ovalbumin. (a) Far-ultraviolet region; solid line: native ovalbumin in pH 7.5 borate (0.05 M) or pH 6.0 phosphate (0.1 M) buffer; dashed line: 5.6 tyrosine residues acetylated (0.05 M borate buffer, pH 7.5). (b) Near-ultraviolet region: native protein and derivatives with varying degrees of tyrosine acetylation (0.05 M borate buffer, pH 7.5).

cyanuric fluoride at six pH values from 9.3 to 11.9 at 25° and at five pH values at 4°. The number of reactive tyrosine residues at 25° was 2.4 at pH 9.3, 2.8 at pH 10.0, 3.4 at pH 10.5, 3.9 at pH 11.0, 5.0 at pH 11.45, and 5.9 at 11.9; at 4° the respective values were 1.9, 2.3, 3.0, 3.4, and 4.0 (pH 11.9 was not measured). On the basis of these results, pH 9.3, 10.0, 11.0, and 11.45 were selected for running concentration curves. Although ovalbumin does not seem to undergo any conformational changes up to pH 11.0 (Cannan *et al.*, 1941) or 11.6 (Stevens and Tristram, 1959) or even pH 12 (Crammer and Neuberger, 1943) in aqueous solution, the presence of 10% dioxane affects its stability. Some conformational changes manifest themselves already at pH 11.5 as shown by the appearance of time-dependent ionization. Since cyanuration is carried out in 10% dioxane, one has to consider what effect it might have upon the conformational state of ovalbumin at pH 11.45 and, consequently, upon the results of cyanuration. It seems justified to assume that the conformational state of ovalbumin at this pH in the presence of 10% dioxane should differ little from that in buffer alone for the first 2 min since 2-min titration values for the two conditions are identical. Since cyanuration of tyrosine residues is known to be very rapid (Gorbunoff, 1967), the time-dependent change of conformation and its possible effect upon tyrosine residue reactivity need not be considered. Therefore, the conformational state of ovalbumin at pH 11.45 under conditions of cyanuration can be assumed to be very close to the state assumed by the molecule at this pH in buffer alone.

Concentration curves were run at 4° at pH 9.3 and at 25° at pH 10.0, 11.0, and 11.5. This gives plateaus of two, three, four, and five groups, respectively, as shown on Figure 1.

The acetylation of ovalbumin with *N*-acetylimidazole was carried out at molar ratios of protein to *N*-acetylimidazole of 1:80–1:700. This resulted in the acetylation of 0.8–2.0 groups. Above this ratio, acetylation continues at a slower rate with a total of eight groups reacting.

The circular dichroism spectra of native ovalbumin and acetylated ovalbumin in pH 7.5, 0.05 M borate buffer are shown on Figure 2a. In the far-ultraviolet region, no changes occur in the circular dichroism spectrum, up to an average acetylation of 5.6 groups, indicating that no important changes in conformation are brought on by the modification of that number of tyrosines. The spectrum of a protein sample with 8.2 groups acetylated displayed a diminution of 5–10% in intensity from that of the native protein, while the positions of the extrema had not changed significantly.

At higher wavelengths, where the bands are due mainly to transitions of aromatic amino acids and disulfide bridges, acetylation results in qualitative as well as quantitative changes, as shown on Figure 2b. The circular dichroism spectrum of native ovalbumin in this region is characterized by a number of overlapping weak bands, the signal remaining positive between 255 and 300 mμ. A weak negative band is evident at 303–305 mμ. Acetylation results in a progressive change toward negative absorption below 290 mμ. It is striking, however, that most extrema in the spectrum remain at essentially the same wavelengths. The one exception is the disappearance of a positive maximum at 286–287 mμ. Fur-

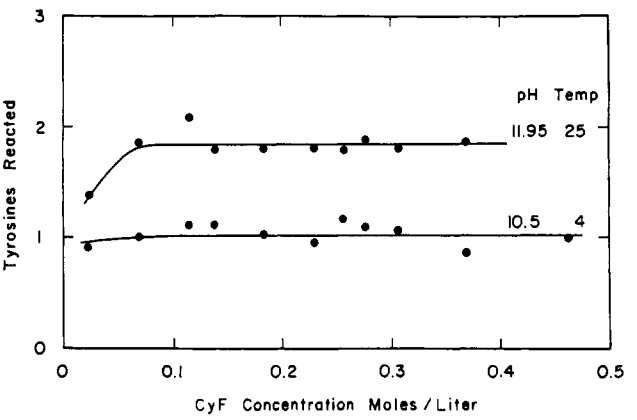

FIGURE 3: Dependence of chymotrypsinogen tryosine reactivity upon cyanuric fluoride concentration at 4 and 25°. Chymotrypsinogen concentration was 6 mg/ml.

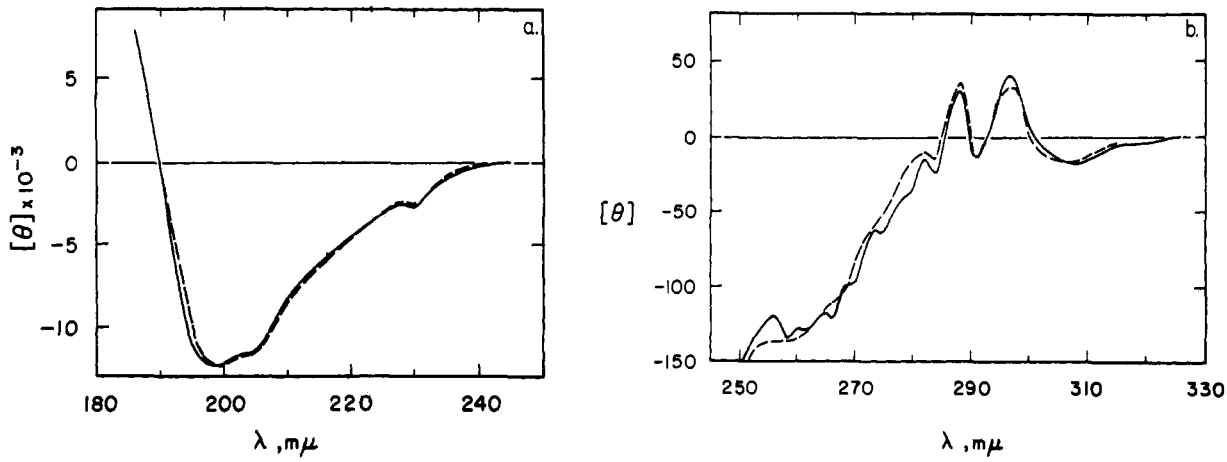

FIGURE 4: Circular dichroism spectra of chymotrypsinogen. Dashed line: native protein in 0.001 M HCl; solid line: 3.2 tyrosine residues acetylated (0.05 M borate buffer, pH 7.5). (a) Far-ultraviolet region; (b) near-ultraviolet region.

thermore, the positive band at 292–293 mμ and the negative band at 303–305 mμ remain essentially unchanged.

Chymotrypsinogen. Chymotrypsinogen contains four tyrosine residues, only two of which are titrated in the native state. The titration curve of chymotrypsinogen in 1 M $KHCO_3$ buffer containing 10% dioxane showed no significant differences from those reported in the literature (Tanford, 1962; Marini and Wunsch, 1963; Inada *et al.*, 1964).

Preliminary experiments were carried out with 0.232 M cyanuric fluoride at six pH values from 9.4 to 12.3 at 25° and at five pH values at 4°. The number of reactive tyrosine residues at 25° was 0.4 at pH 9.4, 0.8 at pH 10.0, 1.2 at pH 10.5, 1.6 at pH 11.5, 1.8 at pH 11.9, and 1.9 at pH 12.2; at 4°, the respective values were 0.3, 0.8, 1.0, 1.5, and 1.4 (pH 12.2 was not measured). Concentration curves were run at 4° at pH 10.5 and at 25° at pH 11.95. pH 10.5 must represent the native state of chymotrypsinogen (Neurath *et al.*, 1956) while pH 11.95 represents that after the onset of irreversible denaturation (Tanford, 1962; Neurath *et al.*, 1956). The results are shown in Figure 3. At pH 10.5, a plateau is obtained at a level of one tyrosine group, at pH 11.95 at a level of two groups.

Acetylation of chymotrypsinogen with *N*-acetylimidazole was carried out over a wide range of concentrations of the acetylating reagent. However, only derivatives acetylated to the extent of 2.5–3.2 tyrosine residues could be prepared. At lower *N*-acetylimidazole concentrations, the samples became turbid during the dialysis required for the removal of the imidazole, while at higher concentrations, the reaction mixture became turbid during the course of acetylation.

The circular dichroism spectra of native and acetylated chymotrypsinogen in the two spectral regions are shown on Figure 4. It is evident that no changes occur in either spectral region of interest when 3.2 tyrosines are acetylated. In the far-ultraviolet region, both the native protein and the acetylated derivative have circular dichroism spectra, characterized by broad negative absorption between 190 and 240 mμ. A negative maximum occurs at 198 mμ, a shoulder at 205 mμ, a broad shoulder in the region of 220 mμ, and a weak negative band at 230 mμ. In the higher wavelength region, the spectrum displays several overlapping bands with positive maxima at 297 and 288 mμ, negative maxima at 291 and 284 mμ, broad negative absorption between 300 and 325 mμ, maximal at 306 mμ, and with a shoulder at 320 mμ. Below 280 mμ, the absorption becomes progressively more negative, with much detail being evident in the spectrum, but no clear band positions may be selected.

Trypsinogen. Trypsinogen contains ten tyrosine residues, of which four groups titrate reversibly with an apparent pK of 10.4, ΔH = 7.9 kcal/mole, and ΔS = −21.2 cal/deg mole, while the other six are buried to different extents (Smillie and Kay, 1961). The titration curve of trypsinogen in 1 M $KHCO_3$ buffer containing 10% dioxane showed no significant differences from that reported in the literature (Smillie and Kay, 1961; Delaage and Lazdunski, 1965a).

Preliminary experiments with 0.232 M cyanuric fluoride were carried out at six pH values from 9.4 to 12.0 at 4 and 25°. The number of reactive tyrosine residues at 4° was 2.0 at pH 9.4, 3.0 at pH 10.0, 4.0 at pH 10.5, 4.2 at pH 11, 5.2 at pH 11.4, and 6.4 at pH 12.0; at 25° the respective values were 3.0, 4.0, 4.9, 5.0, 6.6, and 6.9. Thus, there is a constant difference of about one group (one at pH 9.4 and 10.0, 0.9 at pH 10.5, and 0.8 at pH 11.0) between the reactivity of tyrosine residues at the two temperatures up to pH 11.0 as shown on Figure 5.

The sedimentation and viscosity data at 10° indicate that the hydrodynamic volume of trypsinogen increases reversibly above pH 10 (Smillie and Kay, 1961). The expansion of the molecule cannot be accompanied by changes in secondary structure, however, since there is no change in optical rotation up to pH 11–11.5 (Lazdunski and Delaage, 1967; Neurath *et al.*, 1956). Above pH 11.5, the pH dependence of optical rotation suggests a complete breakdown of native structure (Neurath *et al.*, 1956).

In view of this knowledge, pH 9.4 at 4° and pH 9.4, 10.0, 11.0, and 12.0 at 25° were selected for running concentration curves. pH 9.4 could be considered to represent trypsinogen in the native state; pH 10.0 could represent either the native state or that at the onset of the molecular expansion, while pH 11–11.5 should be its upper limits before the onset of major changes in secondary structure. pH 12.0 could represent trypsinogen in an irreversibly denatured state. Furthermore, in order to verify the effect of temperature upon tyrosine residue reactivity, which was shown by preliminary results, cyanuration experiments were carried out at 4° at pH values of 10.0 and

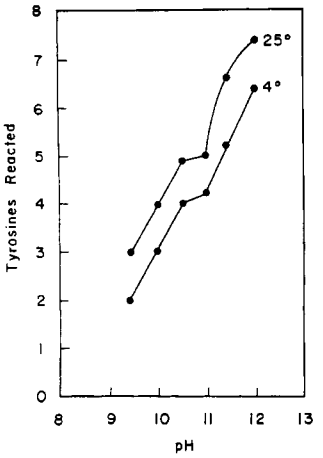

FIGURE 5: Reactivity of trypsinogen tyrosines toward cyanuric fluoride at 4 and 25° as a function of pH. Concentration of trypsinogen was 6 mg/ml; cyanuric fluoride concentration was 0.23 mole/l.

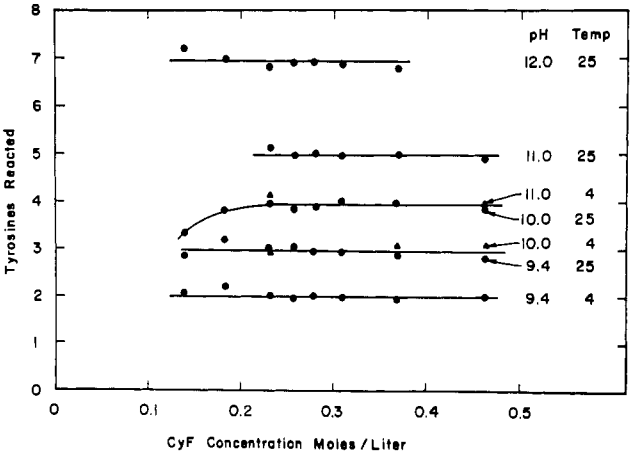

FIGURE 6: Dependence of trypsinogen tyrosine reactivity upon cyanuric fluoride concentration at 4 and 25° as a function of pH. Trypsinogen concentration was 6 mg/ml.

11.0 at the two extreme cyanuric fluoride concentrations (0.232 and 2.32 M). The results are shown on Figure 6. At pH 9.4 and 4° there is a plateau at two tyrosine residues. The next level of three tyrosine residues can be obtained by either raising the temperature to 25° or raising the pH to 10.0. There is a plateau at four tyrosine residues which can be obtained either at pH 10.0 and 25° or at pH 11.0 and 4°; a plateau at five residues can be obtained at pH 11.0 and 25°. The highest reactivity of seven residues is obtained at pH 12.0 and 25°.

Acetylation of trypsinogen with *N*-acetylimidazole was carried out at molar ratios of protein to *N*-acetylimidazole of 1:70–1:700. This resulted in the acetylation of 1.9–4.0 groups. Above this ratio, acetylation of the fifth group continues at a much slower rate. Furthermore, the first four residues acetylate at different rates; three are more reactive than the fourth one. Similar to the case of chymotrypsinogen, the circular dichroism spectra of native trypsinogen and trypsinogen with 3.9 groups acetylated displayed essentially no differences over the entire spectral range examined.

Discussion

Ovalbumin. The reaction of ovalbumin with cyanuric fluoride shows that only five tyrosine residues are accessible to this reagent, while eight are accessible to *N*-acetylimidazole; there are 5.8 tetranitromethane-reactive groups (Sokolovsky *et al.*, 1966). Furthermore, results of cyanuration and acetylation indicate that there is a wide variation of tyrosine residue accessibility to these reagents. This is compatible with the titration data of eight buried groups with an intrinsic p*K* of 9.8 which can be either tyrosine or lysine (Harrington, 1955). Therefore, the tyrosine residues of ovalbumin may be subdivided on the basis of their accessibility to modifying reagents (from highly reactive to unreactive) into seven classes of 2 + 1 + 1 + 1 + 1 + 2 + 1 residues. The first five are accessible to all three modifying reagents with no more than two residues completely (or almost completely) exposed to the solvent medium (Crammer and Neuberger, 1943). One residue is accessible only to tetranitromethane and *N*-acetylimidazole, two residues are accessible only to *N*-acetylimidazole, and one residue is not accessible to any reagent.

Comparison of the circular dichroism spectra of native with acetylated ovalbumin indicates that acetylation of five to six groups can occur without appreciable changes in the circular dichroism spectrum below 250 mμ. The small changes found on acetylation of eight residues suggest that such an extent of reaction is accompanied by some unfolding of the protein molecule. Furthermore, the large excess of reagent required to bring on the acetylation of eight tyrosines can be expected to result in the acetylation of other reactive groups, as well, for example, of lysines. Thus, the charge configuration and chemical constitution of the protein are significantly changed at that extent of acetylation. It is surprising, therefore, that no major changes in conformation occur. The circular dichroism spectrum of ovalbumin with negative maxima at 209 and 222 mμ and a positive maximum at 192 mμ suggest a secondary structure with 25–30% α helix (Timasheff and Gorbunoff, 1967) which is in good agreement with optical rotatory dispersion data (Jirgensons, 1966). The position of the positive band at 192 mμ and the greater intensity of the 222-mμ band than the 209-mμ band might suggest the presence of some β structure in the polypeptide chain fold of ovalbumin (antiparallel β structure is known to have a positive band at 196 mμ and a negative one at 217 mμ (Townend *et al.*, 1966); the presence of that structure, therefore, would shift an α-helical spectrum in the direction observed). Ovalbumin does not have any disulfide bonds stabilizing its secondary structure. It is possible, therefore, that even after acetylation of a significant number of polar groups, the conformation of large portions of the molecule, for example, of α-helical regions is retained. Such regions could become somewhat displaced with respect to each other in order to accomodate the new charge distribution of the molecule. This should redistribute the hydrophobic interactions that stabilize the native structure of ovalbumin, while retaining a favorable over-all free energy of stabilization without major changes in the secondary structure.

The higher wavelength part of the spectrum is characterized by a large number of overlapping weak bands. Acetylation results in a general shift to negative absorption; the positions

of the extrema, however, remain unchanged, except for one. This suggests that the preponderance of the spectral details stems from chromophores other than the most accessible tyrosine residues. The major change in the spectrum consists in the disappearance of a positive maximum at 287 mμ and the appearance of a negative one at 280 mμ. In view of the complexity of the spectrum, however, these cannot be regarded as exact band positions; thus, the 287-mμ maximum reflects probably a band located at a somewhat lower wavelength. Since this change occurs gradually with progressive acetylation, it may be related to changes in tyrosine transitions resulting either directly from acetylation or from changes in the charge environment of the groups or small changes in their three-dimensional space coordinates. It is striking that the bands at 292–293 and 303–305 mμ remain invariant during acetylation. They may be assigned to tryptophan residues (Stevens *et al.*, 1968); furthermore, the presence of this pair of bands, one positive and the other one negative, might suggest exciton splitting due to interactions between tryptophan rings; ovalbumin contains three tryptophans. The lower wavelength bands may be related either to tyrosines or tryptophans, with a phenylalanine contribution below 270 mμ, since ovalbumin contains 21 phenylalanine residues. The absence of disulfide bridges in this protein precludes their contribution to the circular dichroism spectrum.

Chymotrypsinogen. The reaction of chymotrypsinogen with cyanuric fluoride shows that no more than two tyrosine residues are accessible to this reagent in the native or the denatured state. Reaction of chymotrypsinogen with *N*-acetylimidazole results in acetylation of 2.5–3 tyrosine residues. Therefore, on the basis of reactivity with cyanuric fluoride and *N*-acetylimidazole, it appears that the four tyrosine residues of chymotrypsinogen differ in the extent of their esposure.

These results seem to be in good agreement with literature data on the variation of tyrosine residue reactivity in chymotrypsinogen. The titration data (Tanford, 1962) suggest that, of the two reversibly titrating residues, only the one with a pK of 9.7 can be considered as normal (completely accessible to solvent); the second one, with a pK of 10.6, must be partially inaccessible. The presence of one tyrosine which is more reactive than the other three is further suggested by the predominant iodination of only one tyrosine residue at pH 2.4–8.5; iodination becomes random in concentrated urea (Glazer and Sanger, 1964). Reaction with acetic anhydride results in the acetylation of only the two reversibly titrating residues (Oppenheimer *et al.*, 1966). Therefore, it would seem that it is the pK = 9.7 tyrosine residue which can be iodinated, acetylated with acetic anhydride and *N*-acetylimidazole, and reacted with cyanuric fluoride at 4° and pH 10.5. The second tyrosine residue, although as easily accessible to acetylation with various acetylating reagents, can be reacted with cyanuric fluoride only after some loosening of the structure. The difference between the two buried groups is suggested only by the reaction with *N*-acetylimidazole which results in the acetylation of the three tyrosine residues. What is particularly interesting is the fact that acetylation of the third group does not affect the gross conformation of the molecule as shown by the circular dichroism spectrum (Figure 4). This spectrum, with negative maxima at 198 and 205 mμ, suggests a preponderance of unordered structure in this protein (Timasheff and Gorbunoff, 1967); the 230-mμ band is due probably to a disulfide or an aromatic side-chain transition. The high-wavelength end of the spectrum is not significantly altered upon acetylation of three tyrosine residues; this region seems to be determined principally by transitions of the eight tryptophans in the molecule. The positive bands at 288 and 297 mμ and the negative band at 308 mμ are at proper positions for such an assignment (Stevens *et al.*, 1968); in the case of this protein, however, disulfide transitions may also contribute in this region, and the weak negative rotation at 320 mμ may well reflect such groups (Beychok, 1967).

It seems worthwhile to look at the changes in the physical and chemical properties which arise during the activation of chymotrypsinogen to the enzyme. The zymogen → enzyme transformation is accompanied by a decrease in specific levorotation (Neurath *et al.*, 1956). There are no significant changes in size or shape of the molecule in solution (Krigbaum and Godwin, 1968). In general no gross structural differences exist between the zymogen and enzyme and the polypeptide chains seem to follow folding patterns, which are similar within an error of 3 Å in locating the α-carbons of the residues in chymotrypsinogen (Matthews *et al.*, 1967, 1968; Kraut *et al.*, 1967; Sigler *et al.*, 1968; Wright *et al.*, 1968). Nuclear magnetic resonance studies, on the other hand, do suggest that some structural differences exist between the two proteins (Hollis *et al.*, 1968). The small conformational changes which occur during activation must affect the environment of tyrosine residues, since a chymotrypsinogen–chymotrypsin difference spectrum shows a strong band at 287 mμ (Chervenka, 1957, 1959; Benmouyal and Trowbridge, 1966). Such a difference spectrum could reflect either a change in the polarity of the environment of the tyrosyl groups or a change in the charge configuration in its vicinity (Wetlaufer, 1962). On activation, tyrosine 146 becomes the C-terminal group of the B chain. Thus, both effects take place with respect to this group: removal of residues 147 and 148 should make it more accessible to solvent, while the appearance of an α-carbonyl on the same residue changes its charge environment. Titration studies (Tanford, 1962; Havensteen and Hess, 1962; Inada *et al.*, 1964) indicate for both proteins the presence of two tyrosines with different pK's; there are two buried tyrosine residues.

Chemical evidence available in the literature does not suggest any differences between the states of tyrosine residues of chymotrypsinogen and α-chymotrypsin. Iodination results in both cases in the preferential iodination of one tyrosine (Glazer and Sanger, 1964; Dube *et al.*, 1964, 1966), which has been identified as tyrosine 146 in α-chymotrypsin. The second most accessible group to iodination is Tyr 171 (Matthews *et al.*, 1968). Acetylation of α-chymotrypsin with *N*-acetylimidazole was reported to affect two tyrosine residues (Riordan *et al.*, 1965); two residues are acetylated in chymotrypsinogen with acetic anhydride (Oppenheimer *et al.*, 1966).

It has been found in our studies that acetylation of α-chymotrypsin can be carried to the limit of four tyrosine residues depending on the amount of *N*-acetylimidazole used (M. J. Gorbunoff, unpublished data); with chymotrypsinogen, acetylation does not go past 3.2 tyrosines without concomitant denaturation (see Results). Furthermore, the acetylation of tyrosine residues does not seem to affect the conformation in either case. The circular dichroism spectra of α-chymotrypsin with four residues acetylated or of chymotrypsinogen with three residues acetylated are essentially identical in the conformational region with those of the native proteins.

The present work with cyanuric fluoride provides support

from chemical evidence for the difference spectral studies, which have shown that the environment of tyrosine residues changes during the zymogen → enzyme activation. Hachimori *et al.* (1965) have reported that in α-chymotrypsin two tyrosine residues could be reacted with cyanuric fluoride at 4° and pH 9.4. In the present study it was found that the two cyanuric fluoride reactive residues of chymotrypsinogen were accessible to cyanuric fluoride at widely differing conditions. Therefore, it appears that the zymogen → enzyme transformation is accompanied by changes in the environment of one residue. Since the most reactive residue in both proteins seems to be Tyr 146, the one acquiring higher reactivity toward cyanuric fluoride on activation is probably Tyr 171. In chymotrypsinogen, this residue is located in a region of poor resolution of the X-ray data (Wright *et al.*, 1968), so that no conclusions can be made about possible small shifts in its coordinates on activation. Speculations concerning changes in the environment of the two other residues are not warranted by the available chemical evidence, although it is evident that activation releases the fourth group to reactivity with acetylimidazole.

Trypsinogen. In trypsinogen seven residues are accessible to cyanuric fluoride, while only five are accessible to *N*-acetylimidazole. Moreover, they exhibit a wide variation in their reactivity toward the two reagents. Thus, on the basis of cyanuric fluoride and *N*-acetylimidazole reactivity, the tyrosine residues of trypsinogen can be subdivided into six classes. There are 2 + 1 + 1 +1 residues which can be distinguished in the native state and two residues which become reactive upon irreversible denaturation. This gradation in reactivity is in good agreement with the tetranitromethane modification results of Kenner *et al.* (1968); these have shown that no more than six tyrosine residues are accessible to tetranitromethane in trypsinogen in the native state; of these, three to four are more reactive. Furthermore, two residues show preferential kinetic reactivity.

At this point, it seems worthwhile to consider how much the trypsinogen-to-trypsin activation affects the physicochemical properties of the zymogen → enzyme pair. One finds that, just as in the case of chymotrypsinogen → chymotrypsin, they seem to be minor. There is a decrease in the specific levorotation (Neurath *et al.*, 1956), but no change in sedimentation coefficient (Delaage and Lazdunski, 1965b). Some changes in the tyrosine residue environment due to the activation are suggested by the trypsinogen → trypsin difference spectrum (Benmouyal and Trowbridge, 1966). In a similar fashion titration studies suggest normalization of one (Lazdunski and Delaage, 1965) or two (Inada *et al.*, 1964) tyrosine residues while *N*-acetylimidiazole acetylation indicates an increase in reactivity from 5 to 6.7 residues (Riordan *et al.*, 1965). Tyrosine modification studies with tetranitromethane, however, do not indicate any difference in tyrosine reactivity between the zymogen and the enzyme (Kenner *et al.*, 1968).

The involvement of tyrosine residues in trypsinogen → trypsin activation is further suggested by cyanuric fluoride studies. Trypsin has been reported to have six cyanuric fluoride reactive residues at pH 9.5 and 5° (Hachimori *et al.*, 1966). In the present studies, trypsinogen was found to have only two cyanuric fluoride reactive residues under the same conditions. Considering, however, that the cyanuric fluoride reactivity of trypsinogen in the native state is of four levels of 2 + 1 + 1 + 1 residues each, which is paralleled by three levels of *N*-acetylimidazole reactivity at 3 + 1 + 1 residues each, it seems justified to suppose that the environment of several tyrosine residues is affected by activation.

A final remark of caution seems desirable. In chemical modification studies, conclusions on the preferential reactivity of given residues are statistical in nature. When not accompanied by sequence determinations, assignments of reactivity to specific groups are based on the accumulation of evidence pointing in the same direction. In the case of cyanuric fluoride modification, the reactivity of specific groups at each level is strongly suggested by the appearance of discrete plateaus under various conditions (Kurihara *et al.*, 1963). Indeed, in the work on insulin, Aoyama *et al.* (1965) were able to identify in sequence studies the specific tyrosines cyanurated at various total degrees of reaction. Furthermore, there exists a striking parallel between cyanuric fluoride reactivity and solvent perturbation spectroscopy of tyrosine residues (Timasheff and Gorbunoff, 1967); the last technique certainly reflects the degree of exposure of discrete groups (Laskowski, 1966).

Acknowledgment

The author wishes to thank Dr. S. N. Timasheff for his interest in this work and, in particular, for the interpretation of the circular dichroism data.

References

Aoyama, M., Kurihara, K., and Shibata, K. (1965), *Biochim. Biophys. Acta 107*, 257.

Benmouyal, P., and Trowbridge, C. G. (1966), *Arch. Biochem. Biophys. 115*, 67.

Beychok, S. (1967), *in* Poly-α-amino Acids, Fasman, G. D., Ed., New York. N. Y., Marcel-Dekker, p 293.

Cannan, R. K., Kibrick, A., and Palmer, A. H. (1941), *Ann. N. Y. Acad. Sci. 41*, 253.

Chervenka, C. H. (1957), *Biochim. Biophys. Acta 26*, 222.

Chervenka, C. H. (1959), *Biochim. Biophys. Acta 31*, 85.

Crammer, J. L., and Neuberger, A. (1943), *Biochem. J. 37*, 302.

Delaage, M., and Lazdunski, M. (1965a), *Biochim. Biophys. Acta 105*, 523.

Delaage, M., and Lazdunski, M. (1965b), *Biochim. Biophys. Acta 105*, 608.

Dube, S. K., Roholt, O. A., and Pressman, D. (1964), *J. Biol. Chem. 239*, 3347.

Dube, S. K., Roholt, O. A., and Pressman, D. (1966), *J. Biol. Chem. 241*, 5664.

Glazer, A. N., and Sanger, F. (1964), *Biochem. J. 90*, 92.

Gorbunoff, M. J. (1967), *Biochemistry 6*, 1606.

Gorbunoff, M. J. (1968), *Biochemistry 7*, 2547.

Hachimori, Y., Kurihara, K., Horinishi, H., Matsushima, A., and Shibata, K. (1965), *Biochim. Biophys. Acta 105*, 167.

Hachimori, Y., Matsushima, A., Suzuki, M., Inada, Y., and Shibata, K. (1966), *Biochim. Biophys. Acta 124*, 395.

Harrington, W. F. (1955), *Biochim. Biophys. Acta 18*, 450.

Havsteen, B. H., and Hess, G. P. (1962), *J. Am. Chem. Soc. 84*, 448.

Hollis, D., McDonald, G., and Biltonen, R. (1968), *Fed. Proc. 27*, 456.

Inada, Y., Kamata, M., Matsushima, M., and Shibata, K. (1964), *Biochim. Biophys. Acta 81*, 323.
Jirgensons, B. (1966), *Makromol. Chem. 91*, 74.
Kay, C. M., Smillie, L. B., and Hilderman, J. A. (1961), *J. Biol. Chem. 236*, 118.
Kenner, R. A., Walsh, K. A., and Neurath, H. (1968), *Biochem. Biophys. Res. Commun. 33*, 353.
Kraut, J., Wright, H. T., Kellerman, M., and Freer, S. T. (1967), *Proc. Natl. Acad. Sci. U. S. 58*, 304.
Krigbaum, W. R., and Godwin, R. W. (1968), *Biochemistry 7*, 3126.
Kurihara, K., Horinishi, H., and Shibata, K. (1963), *Biochim. Biophys. Acta 74*, 678.
Laskowski, M., Jr. (1966), *Fed. Proc. 25*, 20.
Lazdunski, M., and Delaage, M. (1965), *Biochim. Biophys. Acta 105*, 541.
Lazdunski, M., and Delaage, M. (1967), *Biochim. Biophys. Acta 140*, 417.
Lewis, J. C., Snell, N. S., Hirschmann, D. J., and Fraenkel-Conrat, H. (1950), *J. Biol. Chem. 186*, 23.
Marini, M. A., and Wunsch, C. (1963), *Biochemistry 2*, 1455.
Matthews, B. W., Cohen, G. H., Silverton, E. W., Braxton, H., and Davies, D. R. (1968), *J. Mol. Biol. 36*, 179.
Matthews, B. W., Sigler, P. B., Henderson, R., and Blow, S. M. (1967), *Nature 214*, 652.
McKenzie, H. A., Smith, M. B., and Wake, R. G. (1963), *Biochim. Biophys. Acta 69*, 222.
Neurath, H., Rupley, J. A., and Dreyer, W. J. (1956), *Arch. Biochem. Biophys. 65*, 243.
Oppenheimer, H. L., Labouesse, B., and Hess, G. P. (1966), *J. Biol. Chem. 241*, 2720.
Pechère, J. F., and Neurath, H. (1957), *J. Biol. Chem. 229*, 389.
Riordan, J. F., Wacker, W. E. C., and Vallee, B. L. (1965), *Biochemistry 4*, 1758.
Sage, J., and Singer, S. J. (1962), *Biochemistry 1*, 305.
Sigler, P. B., Blow, D. M., Matthews, B. W., and Henderson, R. (1968), *J. Mol. Biol. 35*, 143.
Smillie, L. B., and Kay, C. M. (1961), *J. Biol. Chem. 236*, 112.
Sokolovsky, M., Riordan, J. F., and Vallee, B. L. (1966), *Biochemistry 5*, 3582.
Stevens, F. S., and Tristram, G. R. (1959), *Biochem. J. 73*, 86.
Stevens, L., Townend, R., Timasheff, S. N., Fasman, G. D., and Potter, J. (1968), *Biochemistry 7*, 3717.
Tanford, C. (1962), *Advan. Protein Chem. 17*, 69.
Timasheff, S. N., and Gorbunoff, M. J. (1967), *Ann. Rev. Biochem. 36*, 13.
Townend, R., Kumosinski, T. F., Timasheff, S. N., Fasman, G. D., and Davidson, B. (1966), *Biochem. Biophys. Res. Commun. 23*, 163.
Tramer, Z., and Shugar, D. (1959), *Act. Biochem. Pol. VI*, 235.
Tristram, G. R. (1953), *Proteins 1*, 219.
Wetlaufer, D. B. (1962), *Advan. Protein Chem. 17*, 303.
Wilcox, P. E., Cohen, E., and Tan, W. (1957), *J. Biol. Chem. 228*, 999.
Wright, H. T., Kraut, J., and Wilcox, P. E. (1968), *J. Mol. Biol. 37*, 363.

Sedimentation Equilibrium Studies with Chymotrypsinogen A in Solution at pH 7.9 and $I = 0.03$*

Diane K. Hancock and J. W. Williams

ABSTRACT: By using the sedimentation equilibrium experiment, it has been found that chymotrypsinogen A undergoes an association reaction when it is dissolved in Veronal buffer at pH 7.9 and at an ionic strength of 0.03. A quantitative evaluation of the data would seem to indicate that under these conditions, the reaction is one of indefinite self-association. However, while every known precaution has been taken to obtain data of high precision, the discrete mechanism of a monomer–dimer–trimer process cannot be completely eliminated. A reason is provided to account for the fact that this discrete association model does almost as well in reproducing the experimental data as does the random association mechanism.

In a recent survey report Nichol (1968) has given consideration to the velocity and equilibrium sedimentation behavior of chymotrypsinogen A in the isoelectric region in glycine-buffered solutions. For several reasons it was not possible at the time to complete a really quantitative treatment with reference to a particular nonideal associating system. For instance, an analysis of the data in terms of a monomer–dimer model lacked uniqueness; certain other mechanisms served about as well to account for the observations.

There is no doubt, however, that given proper solution conditions, association processes do exist. Thus more definitive information about the nature of the association was sought. Scrutiny was restricted to the sedimentation equilibrium ex-

* From the Department of Chemistry, University of Wisconsin, Madison, Wisconsin 53706. *Received February 27, 1969.* This research received its financial support from the National Science Foundation (Grant GB 6063).