Exposure of Tyrosine Residues in Proteins. II. The Reactions of Cyanuric Fluoride and *N*-Acetylimidazole with Pepsinogen, Soybean Trypsin Inhibitor, and Ovomucoid*

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ABSTRACT: The state of tyrosine residues (accessible or buried) in pepsinogen, soybean trypsin inhibitor, and ovomucoid has been examined with cyanuric fluoride and *N*-acetylimidazole. Pepsinogen was found to contain fourteen reactive and three unreactive residues, the reactive ones being of different degrees of reactivity. There are 13.5 *N*-acetylimidazole reactive groups. Soybean trypsin inhibitor contains two reactive and two unreactive residues; the reactive residues are of two types; there are three *N*-acetylimidazole reactive residues. Ovomucoid contains four reactive and two unreactive residues.

The reactive ones can be subdivided into three classes of two + one + one residues according to diminishing reactivity; there are six *N*-acetylimidazole-reactive groups.

The investigation of tyrosine residues in proteins has been widely used as a probe of secondary and tertiary structure, ever since it had been recognized that the anomalous behavior of tyrosines in protein molecules relative to tyrosine in small peptides reflects restrictions imposed upon the protein configuration by these structures (Crammer and Neuberger, 1943; Beaven, 1961; Wetlaufer, 1962). This has resulted in the classification of tyrosine residues into normal (fully exposed) and abnormal (buried) on the basis of titration. Since the question at hand is the extent to which a given group is in contact with solvent molecules (Beaven, 1961; Wetlaufer, 1962; Edsall, 1963), there obviously cannot be any rigorous separation into completely exposed and completely buried residues; the intermediate class of residues, neither fully exposed nor fully buried, must be considered as well (Laskowski, 1966).

The concept of partial exposure brings up the question of the size of the probing molecule that will be able to come into contact with the group in question; it should be expected therefore, that one method of probing into the nature of a given residue will not suffice. Only a combination of several approaches could provide information sufficiently broad to make it possible to arrive at conclusions on the nature of the environment of a given group (Timasheff and Gorbunoff, 1967).

At the present time, the only method capable of attacking directly the question of residue exposure is solvent perturbation spectroscopy since it is based on the size of probing perturbant (Laskowski, 1966). The extent of exposure of a residue and its chemical reactivity vary in parallel fashion (Timasheff and Gorbunoff, 1967); it is possible, therefore, to approach this problem from the side of chemical modification. Several reagents suitable for the chemical modification of tyrosine residues have appeared in recent years. These are cyanuric fluoride (CyF)[1] (Kurihara *et al.*, 1963), *N*-acetylimidazole (Riordan *et al.*, 1965), and tetranitromethane (TNM) (Sokolovsky *et al.*, 1966). Studies with the use of these reagents, although not yet very numerous (see Timasheff and Gorbunoff, 1967), demonstrate the complexity of the problem. Ovalbumin and lysozyme may serve as examples. Titration indicates that in ovalbumin about two groups are normal and eight are buried (Crammer and Neuberger, 1943; Harrington, 1955). It has 1.5 *N*-acetylimidazole-reactive groups (Riordan *et al.*, 1965) while 5.6 react with TNM (Sokolovsky *et al.*, 1966) and 2 are accessible to CyF at pH 10 and 3° (Timasheff and Gorbunoff, 1967). In lysozyme, titration studies indicate three tyrosine residues of $pK = 10.8$ (Tanford, 1962) or two normal and one abnormal residues (Inada, 1961); while two residues can be acetylated with *N*-acetylimidazole,[2] three can be nitrated with TNM (Sokolovsky *et al.*, 1966) and two are accessible to CyF (Kurihara *et al.*, 1963). The following generalization seems to be warranted by the available modification results. *N*-Acetylimidazole can be considered mainly as a modification reagent for normal tyrosines, *i.e.*, those having pK_{int} close to 9.6, $\Delta H_{int}^{\circ} = 6$ kcal/mole, and $\Delta S_{int}^{\circ} = -24$ cal/mole per deg (Tanford, 1962); CyF and TNM show a wider range of reactivity and can be regarded as reagents for any reversibly titrating residues. None of the modifying reagents reacts with tyrosine residues which are buried by the criterion of titration, *i.e.*, which

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: CyF, cyanuric fluoride; TNM, tetranitromethane; STI, soybean trypsin inhibitor.

[2] M. J. Kronman, private communication.

titrate only after irreversible denaturation of the protein.

In a previous study, the reactivity with CyF of tyrosine residues in ribonuclease, α-lactalbumin, and β-lactoglobulin has been reported (Gorbunoff, 1967). The states of the tyrosine residues in pepsinogen, soybean trypsin inhibitor, and ovomucoid have been examined with CyF and *N*-acetylimidazole as probing reagents and compared with their titration properties; the results of this study are presented in this paper.

Experimental Procedure

Materials. Cyanuric fluoride, purchased from Hynes Chemical Research Corp. 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 K & K Laboratories and Fisher Scientific Co., was recrystallized from dry benzene and kept dry over calcium hydride. Pepsinogen crystalline (lots PG6GA, PG6JA, PG6LA, PG6KB, and PG6LC), soybean trypsin inhibitor crystalline (lots SI5993, SI6IB, and SI6JA), and ovomucoid (lot OI7HA) were purchased from Worthington Biochemical Corp. The pepsinogen and soybean trypsin inhibitor were used without further purification. In the latter case, Steiner (1966) has reported that no significant differences could be found between commercial and DEAE-cellulose chromatographed material with regard to the reactivity of tyrosine, tryptophan, and histidine residues. Ovomucoid was purified according to the procedure of J. G. Davis (Donovan, 1967)[3] to remove lysozyme, ovoinhibitor, and flavoprotein, all of which are present in the commercial material. The purification consists in adsorption to and elution from carboxymethylcellulose followed by adsorption to and removal from DEAE-cellulose and a second treatment with carboxymethylcellulose.

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 0.1 M phosphate buffer (pH 7) containing 10% dioxane; for pepsinogen, the molar extinction coefficient at 278 mμ (ϵ 56.1 × 10^3) was used (Perlmann, 1964); for soybean trypsin inhibitor, a conversion factor of 1.059 was used at 280 mμ (Wu and Scheraga, 1962); in the case of ovomucoid the optical factor 0.410 = 1 mg/ml (1 cm path) at 277.5 mμ was used (Donovan, 1967). The molecular weight of soybean trypsin inhibitor was taken as 21,500 (Wu and Scheraga, 1962; Steiner, 1966), that of ovomucoid as 29,000 (Lineweaver and Murray, 1947; Donovan, 1967).

Spectrophotometric titrations were carried out in 1 M $KHCO_3$ buffer containing 10% dioxane by difference spectral technique (Wetlaufer, 1962). The ultraviolet spectra were recorded against protein solutions dissolved in 0.1 M phosphate buffer (pH 7.0) immediately after mixing and at 10-min periods thereafter, below pH 13 for pepsinogen and soybean trypsin inhibitor and below pH 12 for ovomucoid. There was no time-dependent change in the optical density. The optical density taken within 3 min after mixing was taken as the final value for pepsinogen and soybean trypsin inhibitor at pH 13 and above (Wu and Scheraga, 1962) and at 85 sec (λ 295.0) for ovomucoid at pH values above 12 (Donovan, 1967). The maxima of the difference spectra were found to be at 297.5 mμ for pepsinogen, 297.0 mμ for soybean trypsin inhibitor, and 295.0 mμ for ovomucoid. The number of ionized tyrosine residues was calculated for soybean trypsin inhibitor and ovomucoid using an extinction coefficient of 2300 for the phenoxide ion (Crammer and Neuberger, 1943; Sage and Singer, 1962); for pepsinogen, the corresponding extinction coefficient was taken as 2540 (Perlmann, 1964).

Reaction with CyF. The procedure used was the same as previously described (Gorbunoff, 1967). The experimental changes adopted in this study were as follows. After addition of CyF, the reaction mixture was allowed to stand for 1 hr. Then the pH was adjusted to 13 in the case of pepsinogen and soybean trypsin inhibitor and to 13.5 in the case of ovomucoid. The ultraviolet spectrum was recorded between 290 and 340 mμ within 3 min after pH adjustment for pepsinogen and soybean trypsin inhibitor and after 85 sec (λ 295.0) for ovomucoid. The ultraviolet spectrum was recorded *vs.* a standard of the same concentration in 0.1 M phosphate buffer (pH 7.0) containing 10% dioxane. Pepsinogen was used at a concentration of 10 mg/ml, soybean trypsin inhibitor at 8 mg/ml, and ovomucoid at 5 mg/ml; the ultraviolet spectra were recorded in a 2-cm path-length cell.

It was found that in the case of pepsinogen and soybean trypsin inhibitor there was no difference in ultraviolet absorption in the 290–340-mμ region between untreated protein dissolved in a phosphate buffer (pH 7) containing 10% dioxane and the CyF-treated protein adjusted to pH 7. In the case of ovomucoid, this was not the case; CyF-treated samples that had been adjusted to pH 7 showed small positive absorption at 295.0 mμ compared with untreated samples. This was introduced into the calculations as a blank correction.

The number of moles of tyrosine residues which had reacted under any given conditions of pH, CyF concentration, and temperature was calculated as in the case of spectrophotometric titration; the optical density at the wavelength of maximal absorption (297.5, 297.0, and 295.0 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. In the case of ovomucoid, the blank was subtracted from the value of the optical density at 295.0 mμ prior to the calculations.

Method of Data Analysis. The tyrosine residues in a protein were classified on the basis of changes in their reactivity with CyF, 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 CyF concentrations were kept

[3] J. W. Donovan, private communication.

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 CyF) were obtained at these pH values. Furthermore, it was found that at these pH values, there was no time-dependent tyrosine ionization either in the native or partly denatured states (pepsinogen at pH 12.1, ovomucoid at pH 10.9). According to the 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).

Results

Pepsinogen. Pepsinogen contains 17 tyrosine residues (Arnon and Perlmann, 1963) which titrate normally with an apparent pK of 10.9, ΔH = 6.3 kcal/mole, and ΔS = −24 cal/mole per deg (Perlmann, 1964). The titration curve of pepsinogen in 1 M bicarbonate buffer containing 10% dioxane showed no significant differences from that reported by Perlmann (1964).

Preliminary experiments were carried out with 0.232 M CyF at five pH values from 9.3 to 12.1 at 25° and at four pH values at 3°. The number of reactive tyrosine residues at 25° was 7 at pH 9.3, 11 at pH 9.7, 13 at pH 10.8, 13 at pH 11.5, and 14 at pH 12.1; at 3°, the respective values were 7, 9, 11.2, and 13.4 (pH 11.5 not measured).

Pepsinogen is known to suffer reversible conformational transitions in the alkaline pH range between pH 8.5 and 11.0. Perlmann (1961) has reported that reversible conformational transitions take place between pH 9.2 and 10.8 with a midpoint at pH 10.0, while Herriot (1962) reported the onset of structural changes at pH 8.5. It has been suggested recently (Frattali *et al.*, 1965) that pepsinogen undergoes at least two reversible conformational transitions which are independent of each other. The first corresponds to that reported by Herriot; it occurs between pH 8 and 10; the second transition corresponds to that reported by Perlmann.

In the light of this knowledge, pH 9.3, 9.7, 10.8, and 12.1 were selected for running concentration curves. pH 9.3 could be considered to represent pepsinogen at the midpoint of the first conformational transition. pH 9.7 should be representative of the conformations close to that at the midpoint of the second transition, with pH 10.8 being its upper limit (Perlmann, 1961; Herriot, 1938, 1962). pH 12.1 could represent pepsinogen in an irreversibly denatured state (Perlmann, 1961; Herriot, 1962; Frattali *et al.*, 1965).

At pH 9.3 and 25°, poorly reproducible results indicating the reactivity of about seven tyrosines were obtained. When the temperature was dropped to 3°, a plateau was obtained at the level of seven tyrosine residues (Figure 1). By raising the pH to 9.7, a second plateau of 9 groups is attained at high CyF concentrations; the reactivity, however, does not stop at this level as indicated by the upturn in the reactivity *vs.* CyF concentration plot (Figure 1). At 25°, the concentration curves at pH 9.7, 10.8, and 12.1 gave plateaus at levels of 11, 13, and 14 groups, respectively (Figure 1).

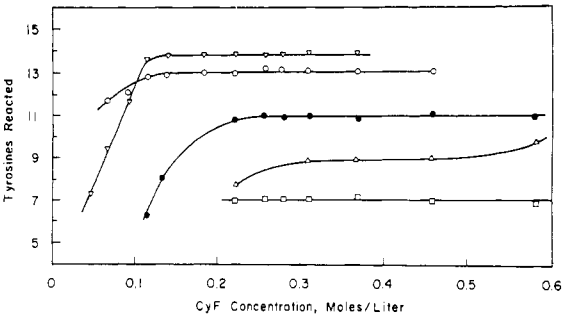

FIGURE 1: Dependence of pepsinogen tyrosine reactivity on CyF concentrations. (□) 3°, pH 9.3; (△) 3°, pH 9.7; (●) 25°, pH 9.7; (○) 25°, pH 10.8; and (▽) 25°, pH 12.1.

Acetylation of pepsinogen with *N*-acetylimidazole was carried out according to Riordan *et al.* (1965) but at a molar ratio above 1:100 in order to determine the maximal number of groups reactive toward *N*-acetylimidazole. The limit of reaction was reached after the acetylation of 13.5 groups accompanied by denaturation of the protein.

Soybean Trypsin Inhibitor. STI contains four tyrosine residues which titrate normally with an intrinsic pK of 9.5 (Wu and Scheraga, 1962). The titration curve of STI in 1 M $KHCO_3$ buffer containing 10% dioxane showed no significant differences from that reported by Wu and Scheraga (1962).

Preliminary experiments were carried out with 0.232 M CyF at six pH values between 9.3 and 12.5 at temperatures of 3 and 25°. At 25°, the number of reactive tyrosine residues is 1.5 at pH 9.3, 1.9 at pH 10.0, 1.9 at pH 10.9, and does not exceed 2 up to pH 12.5. At 3°, the respective values are 1, 1.2, 1.8, and 2. It appears, therefore, that there is a difference in tyrosine reactivity between 25 and 3° below pH 10.9. Since it is known that STI undergoes some reversible conformational changes in the pH range of 9.5–11 at 25° (Steiner and Edelhoch, 1963), pH 9.3 can be taken to be representative of STI in the native state, while any other pH above 9.5 should represent the altered states of the protein. Therefore, concentration curves were obtained at pH 9.3 and 3° and at pH 10.0 and 10.9 at 25°, the last two being within the limits of the reversible conformational change (Wu and Scheraga, 1962; Steiner and Edelhoch, 1963). The results are shown in Figure 2. At 3° a plateau is obtained at a level of 1 tyrosine group at pH 9.3. At 25° a plateau of 1.9 groups is obtained at pH 10 and at the level of slightly over 2 at pH 10.9.

Above the denaturation point, which must be above pH 12 (Wu and Scheraga, 1962; Steiner and Edelhoch, 1963), the reactivity of the tyrosine residues in STI remained at the level of two groups.

STI was treated with *N*-acetylimidazole according to the method of Riordan *et al.* (1965). The starting material for these experiments was purified by passing

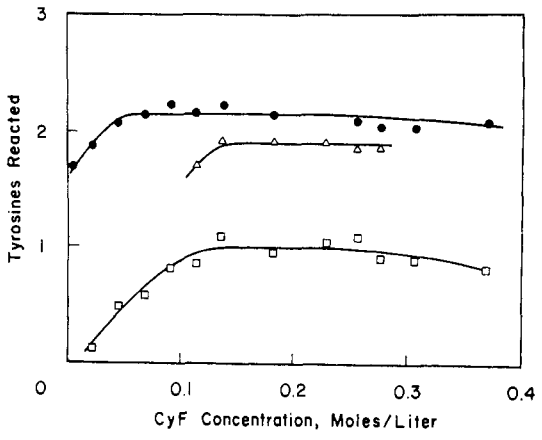

FIGURE 2: Dependence of soybean trypsin inhibitor tyrosine reactivity on CyF concentrations. (□)3°, pH 9.3 (△) 25°, pH 10; and (●) 25°, pH 10.9.

through a Sephadex G-25 column in pH 7.5 0.05 M borate buffer. This eliminated material showing broad absorption between 330 and 300 mμ (sharpened elution pattern). The recovery from the column was 94%. The purified STI showed one major and one minor component in acrylamide gel electrophoresis at pH 9 in 4.5 M urea.[4] It appeared to be identical with the main peak obtained by Steiner (1966) on DEAE-cellulose chromatography of commercial STI. *N*-Acetylimidazole acetylation of STI resulted in the acetylation of two tyrosine residues at a molar ratio of protein to *N*-acetylimidazole of 1:50–170. Above this ratio a slow acetylation of the third group was observed. There was no difference in the extent of tyrosine residue acetylation between the original (commercially available) and the Sephadex-purified STI.

Ovomucoid contains six tyrosine residues which titrate with an apparent p*K* of 12.0 (Donovan, 1967). The titration curve of ovomucoid in 1 M $KHCO_3$ buffer containing 10% dioxane showed no significant differences from that reported by Donovan (1967). This titration curve was calculated from the values of optical density at 295.0 mμ read at 85 sec at pH values above 12 to minimize the time-dependent increase in absorption, not due to ionization of tyrosines (Donovan, 1967).

Preliminary experiments were carried out with 0.232 M CyF at three pH values at 4 and 25°. At 4°, the number of reactive tyrosine residues is 1.5 at pH 9.3, 2.4 at pH 10, and 3.4 at pH 10.9; at 25°, the respective values are 1.8, 2.9, and 3.9. Based on these results and the fact that ovomucoid does not seem to undergo any conformational changes up to a pH of about 10.5 (Jirgensons *et al.*, 1960; Herskovits and Laskowski, 1962a) or even 11.5 (Fredericq and Deutsch, 1949), concentration curves were run at 25° at pH 9.3, 10, and 10.9. pH 10.9 was selected to represent the conformational state after the onset of denaturation, since ovomucoid has been reported to loose activity at pH 11 (Melamed, 1966). This gives plateaus of two, three, and four groups, respectively (Figure 3).

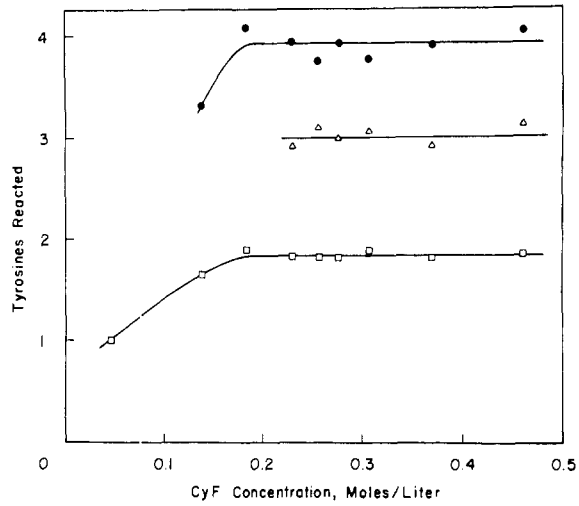

FIGURE 3: Dependence of ovomucoid tyrosine reactivity on CyF concentrations at 25°. (□) pH 9.4; (△) pH 10.0; and (●) pH 10.9.

Ovomucoid was acetylated with *N*-acetylimidazole at a molar ratio of 1:100 to 1:360. This resulted in the acetylation of 0.7–3.0 tyrosine residues. At higher reagent concentrations, acetylation continues slowly until all six groups have reacted.

Discussion

Pepsinogen. The tyrosine residues of pepsinogen exhibit a wide variation in their reactivity toward CyF. At pH 9.3 and 3°, seven tyrosines are found to be reactive. By raising the pH to 9.7, the next level of nine residues is attained, two more residues become reactive by raising the temperature to 25°. At pH 10.8 and 25°, 13 residues are reactive. Above the point of irreversible denaturation which is above pH 11 (Perlmann, 1961; Herriot, 1962; Frattali *et al.*, 1965), the maximal reactivity of 14 tyrosine residues is reached. Although all 17 tyrosines of pepsinogen are found to be normal by the criterion of titration (Perlmann, 1964), they must differ in their extent of exposure to the solvent, since, in the native state, only 8.8 tyrosine residues can be acetylated with *N*-acetylimidazole (Perlmann, 1966) and only 10.4 residues can be nitrated with TNM (Sokolovsky *et al.*, 1966).

As pointed out above, the CyF reactivity of pepsinogen tyrosines was determined at pH values which lie within a region where this protein undergoes reversible conformational transitions. The lowest level of reactivity of seven residues was found at pH 9.3. This lies at about the midpoint of the first conformational transition (about pH 9.4 and 25°). This transition must cause only very minor changes in the protein conformation since it affects almost no changes in the specific rotation, viscosity, or relaxation time; moreover, it is time dependent (Frattali *et al.*, 1965). Considering that the reaction of tyrosine residues with CyF is very rapid and that pepsinogen was cyanurated at pH 9.3 and 3°, it seems fair to assume that its conformational state was rather close to the native state.

[4] I wish to thank Dr. R. F. Peterson for performing the acrylamide gel electrophoresis experiment.

The next three levels in tyrosine residue reactivity were found within the limits of the second conformational transition (see Results). It occurs within the pH range of 9.2–10.8 with a midpoint at pH 10.0 (Perlmann, 1961). This transition is reversible and is accompanied by important changes in viscosity, optical rotation, and relaxation time (Perlmann, 1961; Frattali *et al.*, 1965). The number of reactive residues was 9 at pH 9.7 and 3°; it went up to 11 at 25° and reached 13 at pH 10.8 and 25°. It appears, therefore, that, within the second conformational change, there is a difference in tyrosine residue reactivity between the two temperatures. This difference almost disappears at the upper limit (pH 10.8) of the transition. Since Perlmann *et al.* (1967) have found that the conformational transition of pepsinogen is characterized by a positive enthalpy, it appears reasonable that the tyrosines in question, or at least the protein structure in their immediate vicinity is involved in the conformational change.

On the basis of the CyF reactivity, the tyrosine residues of pepsinogen can be subdivided into six classes. There are four types of residues (7 + 2 + 2 + 2) which can be distinguished within the limits of the two reversible conformational transitions, one residue which becomes reactive upon irreversible denaturation and three residues remaining inaccessible even after denaturation.

This gradation in tyrosine reactivity receives some support from literature data on the nature of the tyrosine residues in pepsinogen. Here 8.8 residues are found to be acetylated with *N*-acetylimidazole (Perlmann, 1966). However, acetylation does not stop at this level. By increasing the molar ratio of the acetylating reagent, the extent of acetylation could be forced up to 13.5 residues, with a concomitant denaturation of the protein. Thus, from 8.8 to 13.5 tyrosine residues are accessible to *N*-acetylimidazole depending upon the experimental conditions. This range of reactivity coincides with that of CyF reactivity of 11 residues at pH 9.7. Since 11 residues are accessible to all three modifying reagents, it is suggestive to consider them as the "free" tyrosines of pepsinogen. Exposure to the solvent of these "free" residues, although great, need neither be complete nor equal. Support for this assumption can be deduced from the results of solvent perturbation experiments. The perturbation of the tyrosines of native pepsinogen caused by 30% propylene glycol is about 37% of that found in 7.7 M urea (Frattali *et al.*, 1965). Although the quantitative evaluation of the tyrosine solvent perturbation spectra is precluded by the typtophan spectra, 37% should be regarded as an upper limit, since it was found that exposure of tyrosine residues in 8 M urea becomes complete only after the splitting of the S–S bridges (Herskovits and Laskowski, 1960, 1962a,b). Since pepsinogen has three disulfide bridges (Arnon and Perlmann, 1963) and its complete amino acid sequence is not yet known, the exposure of chromophores in 7.7 M urea might not represent a maximal value. The 37% perturbation of tyrosine residues can be interpreted at the extreme limits to implicate from six (fully exposed) to twelve (partially exposed) residues (Laskowski, 1966).

In the light of this, the gradation in tyrosine residue reactivity toward CyF might be interpreted as follows. The tyrosine residues of pepsinogen are made up of eleven partially exposed and six buried residues. Seven out of these eleven are more exposed and therefore in better contact with the solvent. This will result in minor differences in the environment of the individual tyrosine residues. Such differences can then be detected by a technique sufficiently sensitive to minor variations in protein topography. Therefore, in the native state 8.8 tyrosines react with *N*-acetylimidazole (Perlmann, 1966), 10.4 with TNM (Sokolovsky *et al.*, 1966), and only 7 with CyF, the 7 being the most exposed residues. As the structure begins to open up, first two and then two more residues become accessible to CyF at pH 9.7, thus, completing the cyanuration of the exposed residues. After a very considerable loosening of the structure, at the end of the second reversible transition, two more tyrosines are liberated. Above pH 11, after the onset of the irreversible breakdown of the molecular structure, one more tyrosine becomes reactive.

Soybean Trypsin Inhibitor. The reaction of STI with CyF shows that no more than two tyrosine residues are accessible to the reagent in the native or the denatured state. Reaction of STI with *N*-acetylimidazole results in acetylation of two or three tyrosine residues depending upon the molar ratio of the reagent. Furthermore, the CyF reactive residues differ in the degree of their reactivity toward this reagent. At 3° and pH 9.3 only one tyrosine residue is reactive. By raising the temperature to 25°, a second tyrosine residue becomes partially reactive. Raising the pH to 10 completes the reactivity of the second group. Therefore, on the basis of reactivity with CyF and *N*-acetylimidazole, it appears that the four tyrosine residues in STI, although normal as defined by titration studies (Wu and Scheraga, 1962), are not identical.

There are some reports in the literature on the variations in tyrosine residue reactivity in STI. It was found that only two tyrosines can be iodinated at pH 8–9 in the native state (Steiner, 1966). Two tyrosine residues are oxidized with tyrosinase at pH 7.5 (Cory and Frieden, 1967). Therefore, it is obvious that the tyrosine residues of STI must be of at least two types. The present study suggests, however, that all four tyrosine residues must be different. The two more reactive residues, which must be those accessible to iodination, tyrosinase oxidation, acetylation with *N*-acetylimidazole at a molar ratio of 1:50 to 1:170, and CyF, show a gradation in reactivity as demonstrated by their reaction with CyF.

Since an increase in tyrosine residue reactivity manifests itself in the pH range of 9.3–10, it can be related to very minor conformational changes found to occur in STI in the pH range from 9.5 to 11 (Steiner and Edelhoch, 1963). Since at pH 9.3 and 25° only 1.5 tyrosines are reactive and the reactivity reaches two residues by pH 10 and remains at that level up to pH 11, it might be assumed that the conformational change affects the environment of one tyrosine residue by making it more (or completely) accessible to the reagent. Furthermore, the fact that, at pH 9.3, it is possible to change the reactivity by half a group by changing the temperature from 25 to 3° suggests that the environments of the two reactive groups are not identical. By imposing greater

TABLE I: The State of Tyrosine Residues in Proteins.

Protein	Titration			*N*-Ac-Imidazole	TNM	CyF	Solvent Perturbation
	p*K*[a]	No. of Reversible	No. of Irreversible				
Lysozyme	10.8[b]	3		2[c]	3[d]	2[f]	2 exposed[e,i]
	10.5, 12.8[g]	2	1				
Zn-insulin	9.6[b]	4		4[h]	2[d]	2 + 1[k]	46% exposed[i]
	10.4, 11.4[g]	3	1				
Zn-free insulin							67% exposed[i]
Ribonuclease	9.9[b]	3	3	3[h]	3[d]	1 + 1 + 1[k]	40% exposed[j]
Pepsinogen	10.9[l]	17		8.8[m]–13.5	10.4[n]	7 + 2 + 2 + 2 + 1	37% exposed[n]
STI	9.5[o]	4		2–3		1 + 1	70% exposed[p]
Ovomucoid	12.0[q]	6		3–6		2 + 1 + 1	60% exposed[q]
							40–60% exposed[r]

[a] The p*K* values reported are either intrinsic or apparent, depending upon the value given in the original publication. [b] Tanford (1962). [c] Kronman, private communication. [d] Sokolovsky *et al.* (1966). [e] Williams and Laskowski (1965). [f] Kurihara *et al.* (1963). [g] Inada (1961). [h] Riordan *et al.* (1965). [i] Weil *et al.* (1965). [j] Herskovits (1965) (T. T. Herskovits and M. Laskowski, to be published). [k] Gorbunoff (1967). [l] Perlmann (1964). [m] Perlmann (1966). [n] Frattali *et al.* (1965). [o] Wu and Scheraga (1962). [p] Steiner (1966). [q] Donovan (1967). [r] Herskovits and Laskowski (1962a).

rigidity on the molecular structure (accentuating differences in reaction rates), the second tyrosine becomes inaccessible to CyF.

A gradation in the reactivity of the two less reactive tyrosine residues, *i.e.*, those which can be iodinated only in 9 M urea (Steiner, 1966), which are affected neither by CyF nor by tyrosinase, is suggested by the *N*-acetylimidazole acetylation of only three tyrosine residues. The results of solvent perturbation studies (Steiner, 1966) which cannot be assessed quantitatively due to the high tryptophan contents of STI, could nevertheless support this point. It was found that about 70% of the tyrosine residues were accessible to propylene glycol. Assuming that the two more reactive residues are almost fully accessible to solvent at room temperature, which would be well supported by their chemical behavior, 70% tyrosine residue exposure would allow for two partially exposed tyrosines. One of these tyrosines could be almost inaccessible to the solvent.

Ovomucoid. The reaction of ovomucoid with CyF shows that only four tyrosine residues are accessible to this reagent, while six residues are accessible to *N*-acetylimidazole. Furthermore the CyF-reactive residues differ in the degree of their reactivity toward this reagent. The lowest level of reactivity, that of two groups, is shown at pH 9.3 and 25°. By raising the pH, two more levels are brought out. At pH 10 three groups are reactive, while at pH 10.9 the maximum of four groups is attained.

Therefore, on the basis of CyF reactivity, it can be claimed that ovomucoid has four types of tyrosine residues. There are two classes of residues (2 + 1) which are reactive in the native state, one residue which becomes reactive with the onset of some conformational changes that affect activity (Melamed, 1966) without causing any significant changes in the structure (Fredericq and Deutsch, 1949), and two residues which remain unreactive.

The information on the nature of the tyrosine residues available in the literature is limited to iodination, acetylation, and fluorescence quenching of phenolic chromophores. Iodination at pH 8 results in the introduction of six atoms of iodine per mole of ovomucoid (Stevens and Feeney, 1963). Whether this means that all tyrosines are accessible to iodine cannot be concluded for lack of detailed information. *N*-Acetylimidazole acetylation resulted in the acetylation of 5.3 residues (Riordan *et al.*, 1965). This value agrees well with the maximal acetylation of six tyrosines reported in the present studies considering that the methods of preparation of ovomucoid were not identical. The strong quenching of fluorescence found in ovomucoid (Teale, 1960; Donovan, 1967) indicates that the phenolic groups are not free, a fact also shown by an apparent p*K* value of 12.0 (Donovan, 1967).

The gradation in tyrosine residue reactivity, demonstrated by CyF, is in excellent agreement with solvent perturbation studies (Herskovits and Laskowski, 1962a; Donovan, 1967). Herskovits and Laskowski have found that the tyrosine residue exposure in ovomucoid at neutral pH was dependent upon the size of the perturbant. Thus 60% of the tyrosines was exposed to 20% dimethyl sulfoxide (small perturbant) but only 40% was exposed to 20% glycerol (bulky perturbant). Donovan, working with D_2O (the smallest perturbant available), found that tyrosine residue exposure was constant in

the pH range of 1.3–7.1, and equal to 60%. Moreover, he concluded on the basis of peak widths that the phenolic groups of ovomucoid were of different degrees of abnormality.

On the basis of the CyF reactivity of 2 + 1 + 1 residues, an exposure of 40–60% of the tyrosine residues can be interpreted to mean that two groups are exposed to the solvent medium to an almost complete extent, while the third and particularly the fourth groups are less exposed. Therefore, it requires some loosening of structure to make the third tyrosine reactive; this is consistent with the fact that the ovomucoid structure tightens up as the pH is lowered (Herskovits and Laskowski, 1962a). The fourth group, the least exposed one, becomes accessible to CyF only after considerable structural changes have taken place.

Thus, on the basis of CyF reactivity and solvent perturbation studies, it is suggested that the tyrosine residues of ovomucoid are made up of four exposed and two buried residues. The exposed residues can be classified into two fully (or almost fully), one partially, and one very slightly exposed.

Conclusions

The high sensitivity of CyF to environmental factors claimed in these studies seems warranted by its ability to complement solvent perturbation studies. The results of tyrosine exposure measurements carried out on a number of proteins by several techniques are shown in Table I. It has been found by solvent perturbation studies that in lysozyme about two-thirds of the tyrosine residues is accessible to solvent (Herskovits, 1965; Williams and Laskowski, 1965), while in insulin, 46% is accessible in the Zn form and 67% in the Zn-free form (Weil *et al.*, 1965). Moreover, in ribonuclease only 40% of the tyrosines was accessible to perturbants to varying degrees (Herskovits, 1965).[5] Studies with CyF showed that in lysozyme two out of three tyrosines were reactive (Kurihara *et al.*, 1963), while in insulin there was a gradation in reactivity. The four tyrosines in insulin could be divided into three classes of 2 + 1 + 1 reactive residues (Kurihara *et al.*, 1963) or 2 + 1 reactive and 1 unreactive residue (Gorbunoff, 1967). The second set of results correlates very well with the solvent perturbation data of 46 and 67% exposure of tyrosine residues (about 2 and 2.7 tyrosines, respectively). In ribonuclease the three reactive tyrosines were found to be different (Gorbunoff, 1967). It must be concluded, therefore, that two out of three tyrosine residues are only partially, and to a varying degree, exposed to the solvent. This is in complete agreement with 40% exposure shown by solvent perturbation studies (Herskovits, 1965).[5] This physicochemical evidence has been borne out by X-ray studies (Kartha *et al.*, 1967; Wyckoff *et al.*, 1967). A similar pattern of correspondence between solvent perturbation and cyanuration is evident also in the case of the three proteins described in this paper.

[5] T. T. Herskovits and M. Laskowski, Jr., to be published.

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On the Localization of Alkaline Phosphatase and Cyclic Phosphodiesterase in *Escherichia coli**

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ABSTRACT: Evidence is presented for the view that alkaline phosphatase, acid phosphatase, and cyclic phosphodiesterase are localized near the surface of *Escherichia coli*, external to the protoplasmic membrane. Phosphate esters which do not penetrate *E. coli* can be hydrolyzed by intact cells, suggesting that these enzymes are outside of the permeability barrier for phosphate esters. However, the per cent of total activity expressed by intact cells (compared with equivalent cell extracts) varies over extremely wide limits, depending upon the substrate and its concentration. It is believed that components of the cell wall are interposed between the enzymes and the external medium. Presumably the different phosphate esters vary in ease of penetration of the wall barrier. A modified procedure for the purification of *E. coli* alkaline phosphatase is also presented.

Alkaline phosphatase is an enzyme formed by *Escherichia coli* when the growth medium lacks P_i; it is P_i repressible (Horiuchi *et al.*, 1959; Torriani, 1960). Malamy and Horecker (1964) discovered that this phosphatase was released into the sucrose medium when *E. coli* was converted into spheroplasts by means of lysozyme and EDTA (Repaske, 1958). Later, it was found that other degradative enzymes were also released on spheroplast formation, including RNase I, 5′-nucleotidase, cyclic phosphodiesterase, and acid hexose phosphatase (Neu and Heppel, 1964a,b, 1965); also DNase I (Cordonnier and Bernardi, 1965); UDPGase and ADPGase (Melo and Glaser, 1966). These same enzymes were also released by a process called osmotic shock in which washed *E. coli* were first exposed to 0.5 M sucrose containing dilute Tris-HCl buffer and EDTA; after this the pellet of cells was rapidly dispersed in "shock medium" consisting of cold water or cold 5×10^{-4} M $MgCl_2$ (Neu and Heppel, 1965; Nossal and Heppel, 1966). The enzymes listed above were selectively released into the shock medium. Many control enzymes were tested and found to remain entirely within the cell during osmotic shock and within the spheroplast during treatment with EDTA and lysozyme.

The selective release suggested that this family of enzymes might be located at or near the surface of the cell. However, examination of broken cell preparations showed almost all of these enzyme activities to be in the supernatant fraction after centrifugation at 100,000*g* for 1 hr, rather than being attached to cell membrane or wall. A consideration of these facts led Malamy and Horecker (1964) to suggest that alkaline phosphatase is localized in the periplasmic space, a region described by Mitchell (1961) between the protoplasmic membrane and the wall layers.

In this paper we present evidence bearing on the localization of alkaline phosphatase, acid hexose phosphatase, and cyclic phosphodiesterase. We also describe a simple procedure for the purification of alkaline phosphatase from shock fluid.

Experimental Section

Materials. Most of the nucleotides and other phosphate esters were Sigma products. Sodium *p*-nitrophenyl phosphate, bis(*p*-nitrophenyl)phosphate, isopropyl β-D-thiogalactopyranoside, and *o*-nitrophenyl β-D-galactopyranoside also were obtained from Sigma Chemical Co., St. Louis, Mo. Uridine 2′,3′-cyclic phosphate, 2′-AMP, and 3′-AMP were from Schwarz BioResearch, Inc., Orangeburg, N. Y. Uniformly labeled [^{14}C]leucine (220 mCi/mmole) was obtained from New England Nu-

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