The Free and Buried Tyrosyl Residues of α_1-Acid Glycoprotein*

K. Yamagami,† Jacqueline Labat,‡ R. S. Pandey, and K. Schmid

ABSTRACT: The free and buried tyrosyl residues of α_1-acid glycoprotein were determined by the chemical techniques of acetylation and nitration using acetylimidazole and tetranitromethane, respectively, and also by spectrophotometric titration. Five of the twelve tyrosyl residues of the native glycoprotein were acetylated while eight were nitrated. In highly concentrated urea or guanidine hydrochloride which leads to a transformation from the β conformation of the native protein to a random coil structure, all tyrosyl residues reacted with both reagents. After removal of the urea, α_1-acid glycoprotein was renatured as judged by the decrease in the number of free tyrosyl residues and the optical rotatory dispersion curve. Hence, the denaturation of this glycoprotein under these conditions is reversible. Spectrophotometric titration showed that five tyrosyl residues dissociated with an apparent pK value of 9.9. Three further residues were characterized by a higher apparent pK value estimated to be approximately 11.1. The remaining four tyrosyl residues showed an abnormal apparent pK value of 11.8. In the presence of 5 M guanidine hydrochloride or after digestion with Pronase, all -yrosyl residues of α_1-acid glycoprotein titrated like the hydroxyl group of free tyrosine.

For the elucidation of the three-dimensional structure of a typical glycoprotein, the study of its topography seems to be of considerable importance as it would reveal the amino acid residues that are located at the surface and those embedded in the interior of the molecule. Moreover, such an investigation should yield information as to the position of the carbohydrate units in relation to some of these amino acid residues.

The present paper, the second report of this series,[1] describes the determination of the free[2] and buried[2] tyrosyl residues of human plasma α_1-acid glycoprotein by chemical and physicochemical methods.

Material and Method

α_1-Acid glycoprotein (orosomucoid) (Schmid, 1953; Weimer *et al.*, 1950; Jeanloz, 1966) was prepared from the supernant solution of Cohn fraction V of outdated, pooled normal human plasma (Bürgi and Schmid, 1961). As reported earlier, this protein, whose molecular weight was assumed to be 44,000 (Jeanloz, 1966), contains approximately twelve tyrosyl and four tryptophyl residues (Bencze and Schmid, 1957).

Chemical Modification of the Free Tyrosyl Residues of α_1-Acid Glycoprotein in the Absence and Presence of Urea. ACETYLATION. Acetylation with acetylimidazole was carried out essentially according to the procedure of Vallee and coworkers (Simpson *et al.*, 1963; Riordan *et al.*, 1965a,b). For each experiment 20 mg of α_1-acid glycoprotein was dissolved in 2.0 ml of pH 7.5, 0.02 M Veronal–HCl buffer containing 1 M NaCl. A 100-fold molar excess of the mentioned reagent dissolved in 0.01 ml of the same buffer (K & K Laboratories, Plainview, N. Y.) over the protein was used. As a control for each set of experiments, α_1-acid glycoprotein was carried through the entire procedure except for the acetylation.

Nitration. The nitration technique of Vallee and coworkers (Sokolovsky *et al.*, 1966; Riordan *et al.*, 1967) was used. TNM,[3] another specific reagent for free tyrosyl residues of polypeptides and proteins, was purchased from Eastman Organic Chemical Co., Rochester N. Y. For each experiment of this series, 20 mg of α_1-acid glycoprotein was dissolved in 2.0 ml of pH 8.0, 0.1 M Tris-HCl buffer. A 100-fold molar excess of TNM diluted with an equal volume of redistilled ethanol over the protein was used. The number of nitrotyrosyl residues was calculated by dividing the molar absorbance of 427 mμ of the modified protein by the molar absorbance of 4200 of nitrotyrosine at 427 mμ (personal com-

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[1] The first report described the measurements of the free and buried tryptophyl residues of this protein (Yamagami and Schmid, 1967).

[2] The terms free or reactive and buried or unreactive tyrosyl residues refer to the tyrosyl residues that are located at the surface of the protein molecule and, therefore, react with certain specific reagents, and to those that are unavailable toward these reagents, respectively.

[3] Abbreviation used that is not listed in *Biochemistry 5*, 1445 (1966), is: TNM, tetranitromethane.

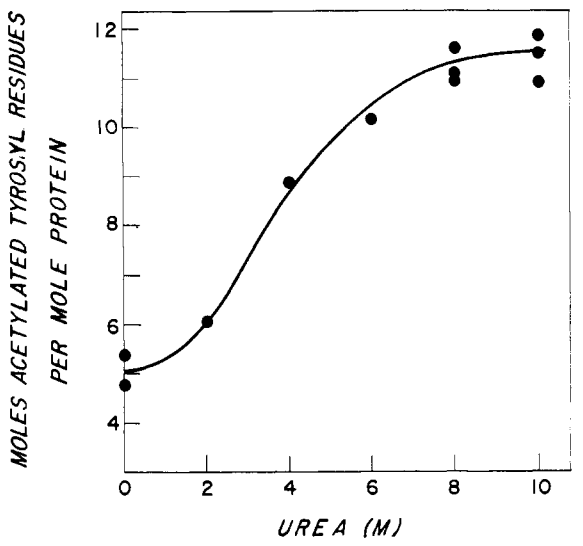

FIGURE 1: The reactive tyrosyl residues of α_1-acid glycoprotein determined by acetylation with acetylimidazole in the presence of increasing concentrations of urea. A 100-fold molar excess of acetylimidazole over protein was used.

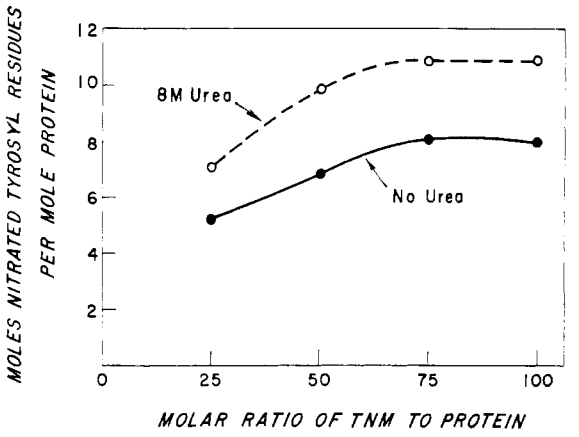

FIGURE 2: Nitration of the reactive tyrosyl residues of α_1-acid glycoprotein in the absence and presence of 8 M urea with increasing molar concentrations of TNM.

munication from J. F. Riordan; see also Riordan *et al.*, 1967). For these absorbance measurements the nitrated glycoprotein was usually dissolved in 0.01 M NaOH and in a few instances in 0.5 M NaCl which was then adjusted to pH 9.3. In both cases identical results were obtained. Both nitration and acetylation were also carried out in presence of urea.

Spectrophotometric Measurements of the Ionized Tyrosyl Residues of α_1-Acid Glycoprotein. For the spectral measurement a Zeiss spectrophotometer (type M4QII) and for the pH determinations a Radiometer pH meter (Model 20) equipped with a calomel electrode K401 and a glass electrode G202B or G202C were used. All experiments were carried out at 23°.

DIFFERENCE SPECTRA. The protein was dissolved in 1.0 M KCl and adjusted to a pH value between 9 and 13. After 2 hr the difference spectra were determined between 250 and 310 mμ, taking a neutral solution of this glycoprotein of the same concentration as reference.

SPECTROPHOTOMETRIC TITRATION. The dissociation of the phenolic hydroxyl groups of α_1-acid glycoprotein was determined at 295 mμ rather than at 244 mμ (Donovan, 1964; Tachibana and Murachi, 1966) by measuring the difference in absorbance at pH values ranging from 7.5 to 13.3. It was assumed that the molar difference in absorbance between the fully ionized and the nonionized *N*-acetyltyrosine at 295 mμ was 2.33×10^3 (Crammer and Neuberger, 1943; Beaven and Holiday, 1952; Donovan, 1964; Tachibana and Murachi, 1966), and that there is the same difference between the ionized and nonionized tyrosine residues of this glycoprotein.

Two different procedures were used for the spectrophotometric titration. Procedure a. A solution of the protein (approximately 0.14%) in 1.0 M KCl was titrated with 10 M KOH increasing the pH by increments of 0.2–0.4 unit until pH 13.3 was reached (first forward titration). Continuous stirring was done with a small magnetic bar. The difference in absorbance was read within 30 sec after the pH was measured. A small aliquot of the protein solution obtained at every pH value was set aside and kept in a covered glass tube for 2 hr after which time the difference in absorbance and its pH were again determined. After the solution was titrated to pH 13.3, it was allowed to stand for 2 hr. It should be noted that the total increase in volume was about 1%. Thereafter, the reverse titration was carried out by addition of small aliquots of 10 N HCl until the pH was 7.5. The second forward titration was done in the same fashion as the first one immediately after the reverse titration was completed.

Procedure b (Tachibana and Murachi, 1966). The glycoprotein solution (1.5 ml, approximately 0.28%) containing 1 M KCl was mixed with an equal volume of the following solutions in order to obtain a desired pH value above 9.5. Carbonate–bicarbonate and KCl–KOH solutions, both of $\Gamma/2$ 0.2, were used to adjust the pH of the protein solution within the ranges from 9.5 to 10.6 and above 10.6, respectively. The ionic strength of these solutions was brought to 1.0 by addition of concentrated KCl solution. The first reading of the difference in absorbance was made within 30 sec after mixing the protein with the appropriate solutions and thereafter at intervals up to 24 hr. Since the solutions were kept in close cuvets, the pH when measured after 24 hr had changed at the most by 0.08 unit.

The obtained titration curves were evaluated as described by Hermans (1962), Tachibana and Murachi (1966), and Tojo *et al.* (1966) according to the equation $\text{pH} - \log [\Delta\epsilon/(\Delta\epsilon_{max} - \Delta\epsilon)] = \text{p}K_{app}$. Further, the product of hydrogen ion concentration and $\Delta\epsilon([H^+]\Delta\epsilon)$ was plotted as a function of $\Delta\epsilon$ to calculate the apparent p*K* value of the different ionized tyrosyl residues from the tangent of the obtained slope (Tachibana and Murachi, 1966). This was done for that part of curve A which does not show any time dependence and also for curve C (Figure 9). The number of tyrosyl residues that ionized according to these p*K* values is indicated by the plateau of the dissociation curves (A′ and D′ in Figure 9).

THE MOLAR RATIO OF TYROSYL TO TRYPTOPHYL RESI-

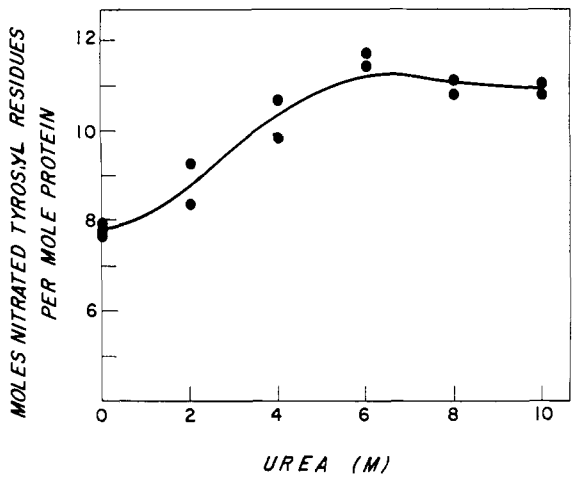

FIGURE 3: The reactive tyrosyl residues of α_1-acid glycoprotein determined by nitration with TNM in the presence of increasing concentrations of urea.

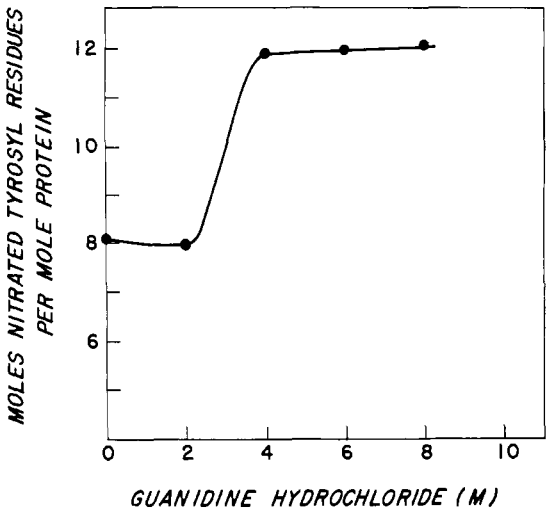

FIGURE 4: The reactive tyrosyl residues of α_1-acid glycoprotein determined by nitration with TNM in the presence of increasing concentrations of guanidine hydrochloride.

DUES. The ratio of tyrosyl to tryptophyl residues of α_1-acid glycoprotein was determined spectrophotometrically by the method of Bencze and Schmid (1957) (Light and Smith, 1963; Tachibana and Murachi, 1966). For these experiments a highly purified glycoprotein preparation made by chromatography on DEAE-cellulose was utilized. An appropriate amount of protein was dissolved in 0.2 M KOH (5.0–5.5 in 5 ml) and after 3 hr the spectrum was measured in the range of 260–310 mμ.

The optical rotatory dispersion was determined with a Cary Model 60 spectropolarimeter. For the *sedimentation velocity analysis* a Spinco Model E ultracentrifuge was employed. Urea (Eastman Organic Chemical Co., Rochester, N. Y.) was recrystallized from 95% ethanol and guanidine hydrochloride (Eastman Organic Chemical Co.) from 50% ethanol at low temperature after removal of impurities by boiling the solution with charcoal.

Results

Acetylation. The number of tyrosyl residues of α_1-acid glycoprotein that reacted with acetylimidazole in the presence of rising concentrations of urea (Figure 1) increased from approximately 5 (absence of urea) to 11.5 (8 and 10 M urea). It is of interest to note that the greatest change in the reacting tyrosyl residues was observed between 2 and 6 M urea. A corresponding observation with regard to the change of specific optical rotation was reported earlier (Schmid and Kamiyama, 1963).

Nitration. Nitration of α_1-acid glycoprotein with increasing concentrations of TNM (Figure 2) revealed that eight tyrosyl residues were substituted above a 75-fold molar excess over the protein. In the presence of 8 M urea and increasing concentrations of TNM, a similar observation was made that approximately 11 tyrosyl residues reacted at the same molar excess of reagent.

Nitration with a 100-fold molar excess of TNM in the presence of increasing concentrations of urea (Figure 3) again showed that approximately eight tyrosyl residues were available to TNM in the absence of urea and essentially all (11.5) tyrosyl residues reacted in the presence of 6 M urea. In the presence of higher urea concentrations, a small decrease in the number of reactive tyrosyl residues was observed. The reason for this decrease is not yet known. An observation which may be comparable was reported by Mihalyi (1965) who noted that the specific optical rotation of fibrinogen exhibited a maximum value in 6 M guanidine hydrochloride and a significant decrease at higher concentrations of this compound. Nitration was also carried out in the presence of increasing concentrations of guanidine hydrochloride, and the results obtained were very similar to those mentioned above (Figure 4).

Ultracentrifugal analysis of α_1-acid glycoprotein in 6 or 8 M urea revealed a symmetrical refractive index curve (Schmid and Kamiyama, 1963). Optical rotatory dispersion measurements (Figure 5) showed that this gly-

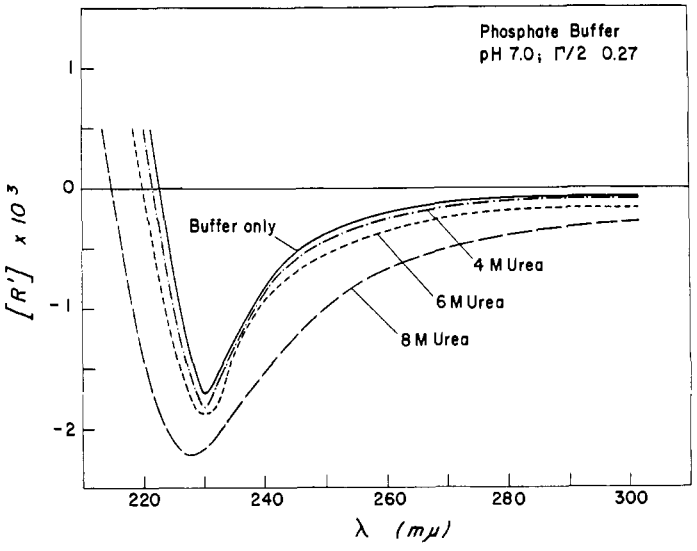

FIGURE 5: Optical rotatory dispersion of α_1-acid glycoprotein in the presence of increasing concentrations of urea.

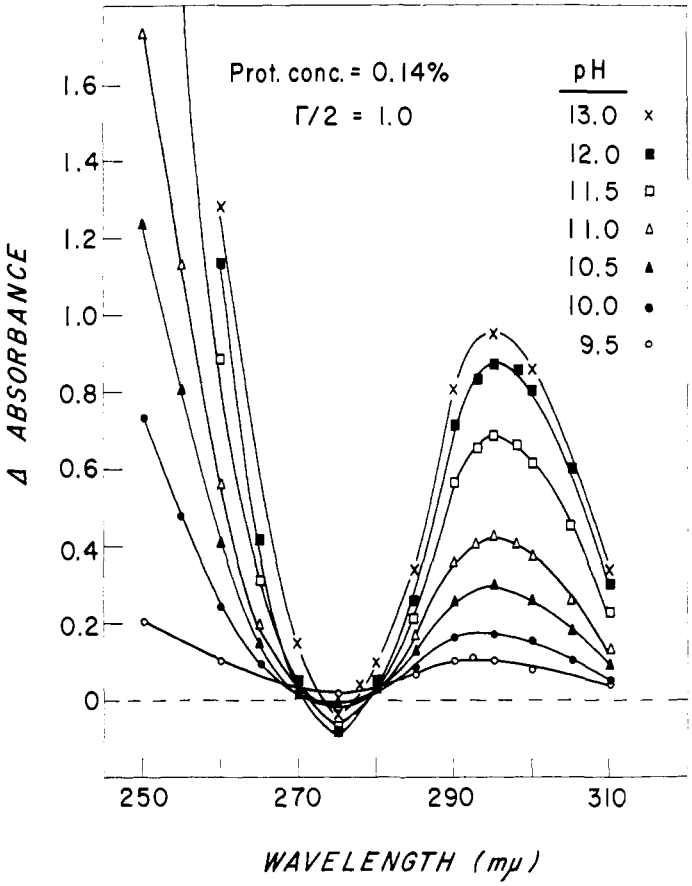

FIGURE 6: Difference spectra of α_1-acid glycoprotein various at pH values.

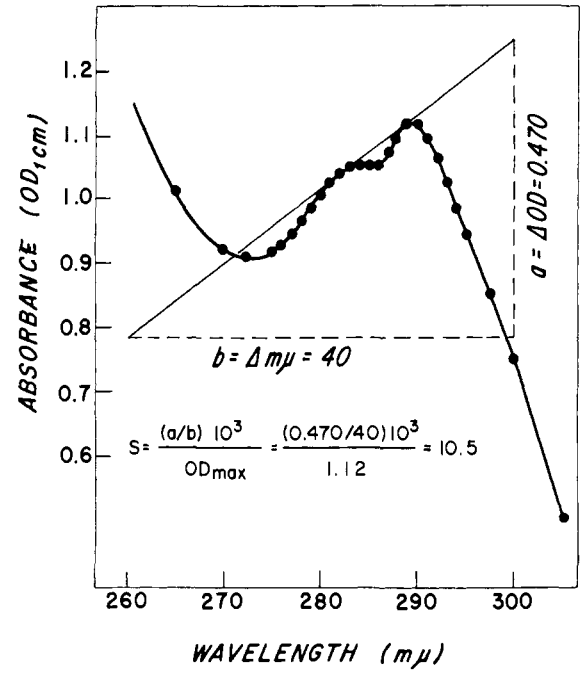

FIGURE 7: Spectrophotometric determination of tyrosyl and tryptophyl residues of α_1-acid glycoprotein (1.02%). From the S value of 10.5 a tyrosine to tryptophan ratio of 2.85 was obtained (Bencze and Schmid, 1957).

coprotein underwent changes in its conformation as judged by the blue shift of the trough. However, a relatively small change was observed when the concentration was increased to 6 M, whereas the major change was noted on increasing the concentration of this reagent to 8 M.

After incubation in pH 7.5, 0.1 M phosphate buffer containing 8 M urea at 25° for 2 hr, α_1-acid glycoprotein was recovered by exhaustive dialysis or passage through a Sephadex G-25 column followed by lyophilization. Nitration of this protein revealed that approximately eight tyrosyl residues reacted with TNM. Acetylation revealed five tyrosyl residues to be free. Moreover, the optical rotatory dispersion curve of the urea-treated protein was identical with that of untreated glycoprotein.

Difference Spectra. The difference spectra of α_1-acid glycoprotein obtained at various pH values are shown in Figure 6. From the maximum of the pH 13 difference spectrum at 295 mμ, it was calculated that this protein contains close to 12 completely ionized tyrosyl residues. This result is in close agreement with the data obtained from the reverse and the second forward titrations of this glycoprotein described below and with the tyrosine content measured by the spectrophotometric procedure of Bencze and Schmid (1957). A molar tyrosine to tryptophan ratio of 2.85 was calculated according to the latter technique (Figure 7). From this value and from the higher of the two observed maxima (Figure 7), it was deduced that this protein preparation probably has an average of 11.5[4] tyrosyl and 4 tryptophyl residues. The latter value agrees with the number of tryptophyl residues determined with Koshland's reagent (Yamagami and Schmid, 1967).

The magnitude of the absorbance of this glycoprotein was found to be time dependent above pH 11 (Figure 8), although this phenomenon was already recognizable at pH values above 10.5 (Figure 9).[5] In the pH range from 11.5 to 12.0 a striking increase in the difference in absorbance started to take place immediately after the protein solution had been adjusted to a pH value within this range. Above pH 12.5, the degree of time dependence became less pronounced probably because the ionization of the tyrosyl residues occurred almost immediately. Any major change observed took place within 30 min (Figure 8).

Ultracentrifugal analyses of α_1-acid glycoprotein incubated at pH 11.5 and 12 in Γ/2 0.1 sodium glycinate for 2 hr showed no denaturation. The sedimentation coefficient of the symmetrical refractive index curve was 2.6 S (1% protein), a value essentially identical with that of native α_1-acid glycoprotein (Schmid *et al.*, 1967). However, at pH 13 denaturation was detected (Yamagami and Schmid, 1967).

Spectrophotometric Titration. The first forward ti-

[4] As shown earlier (Schmid *et al.*, 1967), pooled α_1-acid glycoprotein represents a mixture of polymorphic forms some of which have a lower tyrosine content.

[5] A discrepancy in the pH range of the time-dependent ionization comparable with that found in the experiments presented in Figures 8 and 9 was noted earlier by others (Tachibana and Murachi, 1966).

tration curve (Figure 9, curve A) represents the relationship between the difference in absorbance and the pH measured immediately after the protein solution had been adjusted to the desired values. Curve A shows two marked changes in its slope, namely near pH 10.5 and 11.5, so that this curve may be considered to consist of three parts: (a) below pH 10.5, (b) between pH 10.5 and 11.5, and (c) above pH 11.5. The corresponding pH values obtained 2 hr afterwards yielded curve B. As the time-dependent difference in absorbance was observed at approximately pH 10.5[5] and higher, the curves A and B coincided below these pH values. The products of $[H^+]\,\Delta\epsilon$ plotted against $\Delta\epsilon$ of the first forward titration (Figure 10), derived from the data obtained in the pH range between 8.5 and 10.4 (Figure 9), revealed a PK_{app} of these tyrosyl residues of 9.89. This section of curve A is in good agreement with a first-order sigmoid curve. Based on the above mentioned pK value, a theoretical ionization curve A′ was drawn (A′ in Figure 9 corresponds to A′ in Figure 10) indicating that there are probably 5 tyrosyl residues that dissociate according to this pK.

The curve D is the calculated difference between curves A and A′ and, like curve A, also shows a marked change in its slope near pH 11.5. A theoretical ionization curve (D′ in Figure 9), giving the best fit to curve D between pH 10.5 and 11.5, was drawn and showed that probably three tyrosyl residues are included in this group which dissociate with a pK_{app} of approximately 11.1. The difference between curves D and D′ probably indicates a third group of four tyrosyl residues that dissociate with an even higher pK_{app} value which was found to be approximately 11.8.

The reverse titration data (curve C, Figure 9) were replotted as shown in Figure 10 and indicated a pK_{app} of 10.4, suggesting that all 12 tyrosyl residues of the glycoprotein, which was almost but not completely denatured, dissociate apparently at the same rate. However, when the mentioned reverse titration was carried out immediately after the protein had been denatured very little, the obtained curve was abnormal being located between curves A and C (not shown in Figure 9). The second forward titration curve coincided almost exactly with curve C. If the incubation of this glycoprotein was prolonged and the back-titration was carried out after 24 and 48 hr, the pK_{app} was also found to be 10.4. However, when the protein was dissolved in 5 M guanidine hydrochloride and the forward titration (procedure a) was carried out after 1 hr, a pK_{app} of 10.0 was obtained. Back-titration which followed immediately yielded the same value. Titration of a Pronase digest of this plasma globulin dissolved in 1 M KCl yielded a pK_{app} value of 10.1 for the forward titration and 10.0 for the back-titration which was performed immediately.

Discussion

α_1-Acid glycoprotein contains five freely reacting tyrosyl residues as judged by the specific acetylation and eight phenolic groups that were reactive in the presence of TNM. Hence, the tyrosyl residues of the native form of this glycoprotein can be separated into three groups:

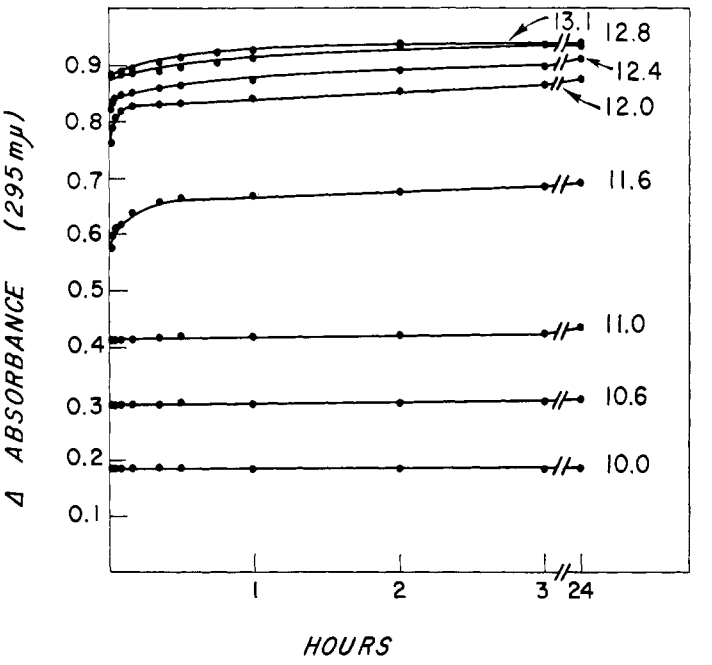

FIGURE 8: Time-dependent difference absorption of α_1-acid glycoprotein measured at 295 mμ and at various pH values. The protein concentration was 0.14%.

(1) five residues that reacted very freely, (2) three residues that appeared slightly buried, and (3) four which were unreactive or completely buried. The latter residues became reactive in the presence of high concentrations of urea and, therefore, are perhaps strongly hydrogen bonded in the native protein. Since native α_1-acid glycoprotein does not undergo any conformational changes between pH 7.5 and 8.0 (Schmid and Kamiyama, 1963) in the pH range in which the acetylation and nitration were carried out, the difference in the number of the reactive tyrosyl residues determined by the mentioned two methods is indeed due to the difference in the reactivity of three tyrosyl residues and not due to partial unfolding of the conformation of the native protein. Comparable differences in the reactivity of the phenolic groups of certain proteins such as carboxypeptidase A, myoglobin, and ovalbumin toward these two reagents were reported earlier (Riordan *et al.*, 1965b; Sokolovsky *et al.*, 1966; Timasheff and Gorbunoff, 1967). The various factors contributing to the differences in the reactivity of one group of amino acid residues of a native protein were recently discussed by Timasheff and Gorbunoff (1967).

In concentrated urea solutions the β conformation of native α_1-acid glycoprotein (Sarkar and Doty, 1966; Yamagami and Schmid, 1967) is transformed into a random structure. This transformation probably explains the availability of all tyrosyl residues in the presence of this reagent. In a corresponding study on the tryptophyl residues of this protein (Yamagami and Schmid, 1967), it was shown that they reacted completely with 2-hydroxy-5-nitrobenzylbromide in the presence of 5 M guanidine hydrochloride, a solvent which also led to the formation of a random coil structure. After removal of urea, the α_1-acid glycoprotein had essentially assumed its native

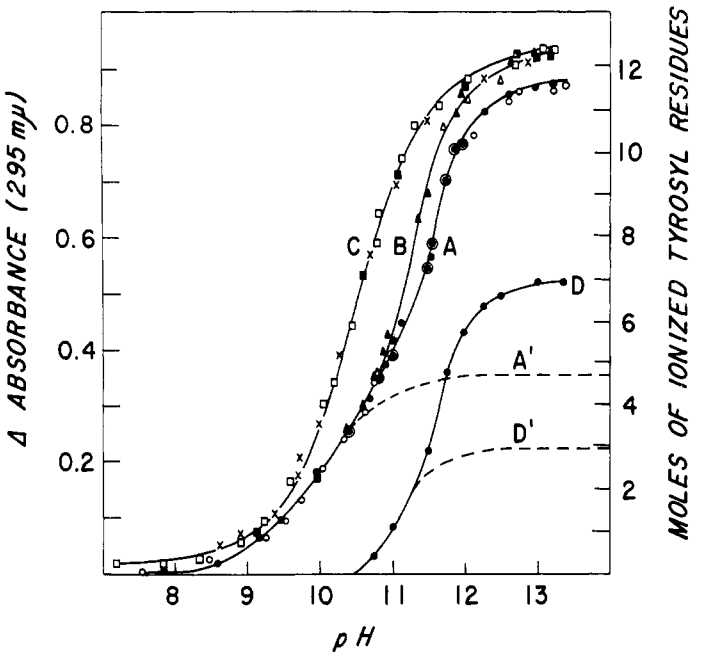

FIGURE 9: Spectrophotometric titration of α_1-acid glycoprotein. Protein concentration 0.14%. (A) (●, ○) First forward titration measured immediately after the pH was adjusted. Results obtained by procedure b are marked with ⊙. (B) (▲, Δ) First forward titration measured 2 hr after the pH was adjusted. (C) (■, □) Reverse titration carried out after 2-hr standing at pH 13. Open signs: data obtained with type C electrode; full signs: data obtained with type B electrode. (C) Second forward titration. For further explanation, see text.

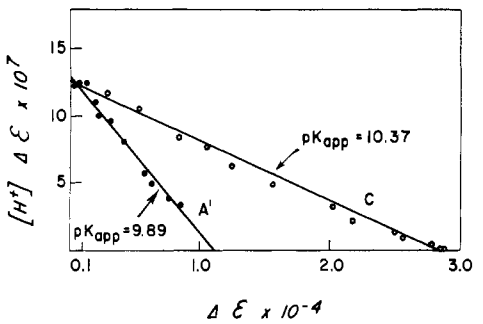

FIGURE 10: Evaluation of spectrophotometric titration curve according to the equation $[H^+]\Delta\epsilon = \Delta\epsilon_{max}K - \Delta\epsilon K$ (Tachibana and Murachi, 1966). (A') First forward titration. Data taken from pH range of 8.5–10.4 of curve A in Figure 9. (C) Reverse and second forward titration. Data taken from pH range of 9.7–13.0 of curve C in Figure 9.

structure, indicating that this conformational transition is reversible.

The spectrophotometric titration of α_1-acid glycoprotein confirmed the presence of three different groups of tyrosyl residues: (1) five residues that dissociated with a pK_{app} value of 9.9, a value close to that of free tyrosine. The dissociation of these residues was not time dependent; (2) three further tyrosyl residues that dissociated according to a pK_{app} of 11.1. The dissociation of these residues is time dependent and, hence, it appears that they are partially buried or located relatively close to the surface of the protein molecule and are perhaps weakly hydrogen bonded. These residues correspond most likely to those which do not react with acetylimidazole, but do react with TNM. It should be added that at pH 11.5 and even at pH 12, the structure of the native molecule is not significantly changed as judged by ultracentrifugal analysis. (3) The remaining four tyrosyl residues that were titrated after partial denaturation of the protein in the very alkaline pH region. It is of interest to note that even a prolonged incubation time of up to 48 hr at pH 13 is not sufficient to release all these tyrosyl residues. Complete denaturation was achieved by incubation in 5 M guanidine hydrochloride or 6 M urea or by digestion of the protein with Pronase as evidenced by pK_{app} values which were essentially identical with those of free tyrosine.

Based on spectrophotometric titration studies several proteins including stem bromelain (Tachibana and Murachi, 1966), hen egg-white lysozyme (Tojo *et al.*, 1966), and bovine heart cytochrome *c* (Hamaguchi *et al.*, 1967) have been shown to possess three groups of phenolic residues with different dissociation ranges. By contrast, fetuin appears to have no buried tyrosyl residues (Verpoorte and Kay, 1966), whereas duck egg-white lysozyme possesses two groups of phenolic residues (Tojo *et al.*, 1966).

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Transformation of Active-Site Lysine in Naturally Occurring Trypsin Inhibitors. A Basis for a General Mechanism for Inhibition of Proteolytic Enzymes*

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ABSTRACT: Protein inhibitors of trypsin generally fall into one of two classes, those requiring a lysine residue for activity and those requiring an arginine residue. Guanidination of four "lysine inhibitors" (lima bean inhibitor and turkey, duck, and cassowary ovomucoids) did not abolish their trypsin-inhibitory activities. It did, however, make them weaker inhibitors. Amidination resulted in almost no detectable inhibitory activity, with an important exception of amidinated lima bean inhibitor which retained definite activity. An "arginine inhibitor" (chicken ovomucoid) was not affected by such treatments. The inhibitors in which the active-site lysine had been transformed into a homoarginine residue, in contrast to the native inhibitors, were now inactivated by treatment with 1,2-cyclohexanedione, a relatively specific reagent for the modification of arginine. From the kinetics of inhibition at different temperatures and different solution viscosities, the rate-determining step in inhibition appeared to be a monomolecular process following formation of the initial Michaelis-type complex. Since the peptide bonds of the lysine derivatives prepared should not be cleaved by trypsin, it was concluded that proteolysis of the inhibitor by the enzyme is not essential for inhibition, and that the rate-limiting step is most probably a conformational change. A general mechanism is proposed for the inhibition of proteolytic inhibitors.

The active site of the inhibitor is a specific amino acid residue for which the enzyme has specificity. In the case of trypsin inhibitors, this would be a particular lysine or arginine. In order for a protein to be an inhibitor two requirements must be met. (a) The enzyme must have a high affinity for the inhibitor at this residue. (b) The peptide bond of this residue is relatively resistant to proteolysis and is cleaved very slowly or incompletely, if at all, by the enzyme.

Modification of the amino groups in several naturally occurring trypsin inhibitors has resulted in a loss of the inhibitory activity (Fraenkel-Conrat *et al.*, 1952; Stevens and Feeney, 1963; Simlot and Feeney, 1966; Haynes *et al.*, 1967). Inhibitors which appeared to have essential lysine residues included lima bean inhibitor, bovine colostrum inhibitor, and ovomucoids of turkey, duck, cassowary, and penguin. By kinetic analysis it was concluded that a specific lysine residue was essential for the trypsin-inhibitory activity of several of the inhibitors studied (Haynes *et al.*, 1967). Soybean trypsin inhibitor and chicken ovomucoid, which are not inactivated by modification of their amino groups, were inactivated by modification of their arginine residues with 1,2-cyclohexanedione (Liu *et al.*, 1968). Since trypsin is fairly specific for the hydrolysis of bonds involving lysine or arginine side chains, and does not readily hydrolyze their derivatives, these observations supported the hypothesis that a specific lysine or arginine residue was essential for the activity of naturally occurring trypsin inhibitors. A general mechanism of action for these inhibitors involving proteolytic cleavage at this specific

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