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REACTIONS OF CYANURIC HALIDES WITH PROTEINS

I. BOUND TYROSINE RESIDUES OF INSULIN AND LYSOZYME AS IDENTIFIED WITH CYANURIC FLUORIDE

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SUMMARY

The ultraviolet absorption band of tyrosine was found to be remarkably lowered and shifted toward shorter wavelengths by treatment with cyanuric fluoride, $(CNF)_3$, whereas the bands of other amino acids were either unaffected by the treatment or affected below 295 mμ, the peak position in the difference spectrum of the ionization of tyrosine. By use of this phenomenon, the reactivities of the tyrosine residues of lysozyme and insulin with cyanuric fluoride were examined, and the following facts were established. Two of the three tyrosine residues in the lysozyme molecule react with cyanuric fluoride and the remaining one is non-reactive. Of the four tyrosine residues in the insulin molecule, two are reactive while the other two are non-reactive. Upon addition of alkali to an insulin solution, one of the two non-reactive residues is transformed rapidly and the other slowly into the reactive type. Of these two bound residues of the non-reactive type one is in the A the other in the B chains of insulin. The numbers and the positions of the bound residues of the same proteins, identified by other properties, are discussed.

INTRODUCTION

Little is known of the pairs of amino acid residues hydrogen bonded or linked by hydrophobic bonding in proteins, whereas we know the pairs of purine and pyrimidine bases hydrogen bonded in nucleic acids. The determination of various states of amino acid residues and their pairing in proteins is of great importance, since this basic knowledge will enable us to describe the tertiary structure in terms of the pairs of residues. To identify the pairs, the numbers of free and bound residues of each amino acid in a protein molecule have to be estimated, and they have to be located in the amino acid sequence. From this view-point, the states of amino acid residues in the proteins, whose primary structures are known, have drawn much attention. In particular, tyrosine residues have been studied most extensively since several

Abbreviation: CyF, cyanuric fluoride.

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spectroscopic techniques are available. These are briefly introduced below as applied to insulin and lysozyme.

The absorption band of tyrosine shifts from 275 to 293 mμ with intensification, when the phenolic group ionizes with alkali into phenoxide ion. CRAMMER AND NEUBERGER[1] were the first to apply this phenomenon to the investigation of tyrosine residues of proteins, and found strongly bound residues in egg albumin, which did not ionize unless the molecule was denatured by strong alkali at pH 13.0. They also observed an anomalously high pK value of 11.0 for the tyrosine residues in insulin as compared with 10.0–10.1 (refs. 2–4) for free tyrosine, but could not differentiate between free and bound residues. This technique has since been applied successfully to various proteins by many workers to distinguish free and bound tyrosine residues or to determine their numbers in the molecule. Recently, INADA[5] in our group measured by the same technique the rate of ionization of the tyrosine residues of insulin at various pH values, and established that one of the four tyrosine residues in the molecule ionized slowly with alkali while the remaining three residues ionized instantaneously. The pK value of the slowly ionizing residue was 11.4 and one hydroxy ion was involved in the ionization. With lysozyme, FROMAGEOT AND SCHNEK[6] observed a reversible transformation of the tyrosine band with a normal ionization curve of the first-order sigmoid shape, while TANFORD AND WAGNER[7] obtained an ionization curve which was flatter than would be expected from the simple ionization mechanism of a single kind of tyrosine residue. The abnormality was confirmed also by DONOVAN, LASKOWSKI AND SCHERAGA[8] for lysozyme in KCl and urea solutions. The ionization curves obtained by INADA[5] immediately and a long time after addition of alkali revealed the fact that one of the three tyrosine residues ionized very slowly with a pK value of 12.8 and two hydroxyl ions were involved in the reaction.

The tyrosine residues of insulin were studied by a different approach by LASKOWSKI, LEACH AND SCHERAGA[9], LEACH AND SCHERAGA[10] and LASKOWSKI, WIDOM, MCFADDEN AND SCHERAGA[11], and the following results were obtained. When a heptapeptide with the B26 tyrosine residue was released from the insulin molecule by tryptic digestion, the absorption spectrum changed, and the difference spectrum showed two peaks at 279 and 286 mμ which were interpreted as due to the cleavage of the hydrogen bond of the B26 residue. The difference spectrum between neutral and acidic solutions of insulin was similar to that observed on tryptic digestion. However, the same type of difference spectrum was obtained between neutral and acidic solutions of the tryptic digest. They concluded from these results that there exist two bound tyrosine residues in the insulin molecule; one is the B26 residue hydrogen bonded to a non-ionizable acceptor, and the other is a residue hydrogen bonded to an ionizable acceptor and freed at an acidic pH. The four tyrosine residues of insulin are at the positions A14, A19, B16 and B26. From a hypothetical model of the tertiary structure, they[9,12] inferred that the residue hydrogen bonded to an ionizable acceptor is the B16 residue. This conclusion accords with the result obtained by DE ZOETEN, DE BRUIN, HAVINGA AND EVERSE[13,14], who found that the rates of iodination of the two tyrosine residues in the B chain are considerably smaller than those observed for the two residues in the A chain.

This series of reports deals with the reactions of cyanuric halides (fluoride and chloride) with various proteins. As demonstrated later, the ultraviolet absorption band of tyrosine is lowered and shifted toward shorter wavelengths by treatment

with CyF resulting in a drop of absorbancy at 295 mμ to practically zero, whereas the absorbancy values at the same wavelength for other amino acids are unaltered by the treatment. By use of this phenomenon, one can differentiate the tyrosine residues of proteins into CyF-reactive and non-reactive types and can determine their numbers in the molecule. Furthermore, spectroscopic measurements of the peptides derived from a CyF-treated protein by enzymic digestion or cleavage of disulfide bonds enable us to locate the two types of residues in the amino acid sequence, since the characteristic tyrosine band only of the non-reactive type remains unaltered in the CyF-treated protein. In the present paper are described the numbers of the two types of residues in the molecules of insulin and lysozyme together with their distribution in the A and B chains of insulin. These numbers are expressed by n throughout this paper. The reactions of $(CNCl)_3$, which has a different specificity with regard to reactions with amino acid, will be reported in a future paper.

EXPERIMENTAL

Materials

CyF was prepared from cyanuric chloride with SbF_3 and $SbCl_5$ by the method of MAXWELL, FRY AND BIGELOW[15]. Crude CyF was redistilled twice; b.p. 74°. Crystalline zinc beef insulin was purchased from Shimizu Pharmaceutical Co., and a recrystallized sample was prepared by the method of ROMANDS, SCOTT AND FISHER[16] as modified by SAWADA, SHIBATA AND ITANI[17]. Lysozyme crystals were prepared from hen egg white by the method of ALDERTON AND FEVOLD[18]. The concentrations of insulin and lysozyme were determined gravimetrically, assuming their molecular weights to be 5734 (ref. 19) and 14 500, respectively, in which the latter value was taken from 14 372–14 714, the value calculated from the amino acid composition[20,21].

The procedure to determine the moles of CyF-non-reactive tyrosine residues

The reactions of CyF with insulin were observed spectroscopically by the following procedure (the procedure was slightly modified for lysozyme as illustrated later). A buffered solution of insulin was prepared by mixing 2 ml of an insulin solution with 6 ml of 1.0 M bicarbonate buffer with KOH of a desired pH value. One ml of a CyF solution in dioxane was added to the buffered sample solution, and the mixture was left standing at 5° for 1.5 h, during which the reaction proceeded to completion and CyF added in excess was hydrolysed into cyanuric acid. As described later in greater detail, the absorption band of CyF-reactive tyrosine residues disappears during the reaction.

To estimate the molar concentration of the residual and CyF-non-reactive tyrosine residues in the reaction mixture, the following difference spectrophotometry was employed. The pH value of the reaction mixture was changed to 12.0–12.5 by addition of 1 ml of a KOH solution, and was incubated at room temperature for 1 h to ionize completely the residual tyrosine residues. The difference in absorbancy at 295 mμ between this alkaline sample mixture and its reference derived from the same reaction mixture by adjusting the pH value to 7.0 was read with a Hitachi spectrophotometer model EPU-2A. The molar concentration of the non-reactive residues was calculated, assuming the difference, $\Delta\varepsilon_M$, in molar absorbancy coefficient at the wavelength to be 2305 (ref. 5). By this difference spectrophotometry, one can

eliminate the effect of overlapping of the bands of other aromatic amino acid residues and their reaction products as explained later with the data obtained on amino acids. In general, the reaction mixture adjusted to pH 7.0 should be the reference to be compared with the alkaline sample mixture. However, this reference mixture was found to be replaceable by a simpler mixture of 8 ml of the sample insulin solution of pH 7.0, 1 ml of dioxane without CyF and 1 ml of water. This is because the non-ionized form of tyrosine residues (Curve C in Fig. 1) and cyanuric acid (Curve B) as the by-product show practically no light absorption at 295 mμ, and because the absorption at the wavelength by other amino acid residues does not change on treatment with CyF.

In the preparation of a sample solution of lysozyme, 1 ml of dioxane was added to 2 ml of a lysozyme solution before mixing with a buffer. Lysozyme solutions at higher pH values are unstable and become turbid, and the addition of dioxane stabilizes them. Another modification was made in the observation of the reaction mixture. As demonstrated by INADA[5], it takes several hours for the bound tyrosine residue of lysozyme to ionize completely with alkali. To accelerate the ionization, the alkaline sample mixture was diluted 2-fold with 8 M guanidine solution, and the same volume of the guanidine solution was added to the reference mixture. The $\Delta\varepsilon_M$ value at 295 mμ in the ionization of tyrosine with 4 M guanidine was found to be 2500, which is slightly higher than the value obtained without guanidine and was used for the calculation of the moles of tyrosine residues in the reaction mixture.

RESULTS

(1) Reactions of CyF with amino acids

CyF is stable in dioxane and shows intense ultraviolet absorption but no peak above 230 mμ (Curve A in Fig. 1), which was the lower limit of observation because of the absorption by dioxane used as the solvent. The longer wavelength end of absorption is approx. 300 mμ, and ε_M at 295 mμ is as low as 0.8. When the solution is mixed with water, CyF is hydrolysed into cyanuric acid, which shows practically no absorption above 280 mμ (Curve B in Fig. 1). In 10% dioxane solution and at room temperature, the hydrolysis was completed within 30 min as judged from the spectral change.

As a preliminary experiment, the reaction of CyF with various amino acids was observed spectroscopically. Curve G in Fig. 1 is the spectrum of the mixture of $2.22 \cdot 10^{-2}$ M CyF and 0.196 mM tyrosine at pH 9.1 in 10% dioxane solution, which was observed after a 1.5-h incubation period at room temperature. In the measurement, the same CyF solution without tyrosine incubated for the same period was used as the reference. By treatment with CyF, the band (Curve D) of non-ionized tyrosine (approx. 90% of the total tyrosine at pH 9.1) is markedly lowered and transformed into a weak band at a shorter wavelength. The maximum of this weak band appears to be slightly below 260 mμ, but could not be determined owing to the strong light absorption by cyanuric acid, the by-product, used as the reference. A similar observation made at pH 12.6 showed a remarkable spectral change from Curve F of ionized tyrosine to Curve H, which is almost identical with the spectrum of the product formed at pH 9.1. These results are shown in Fig. 2 in the form of difference spectra (G—D and H—F).

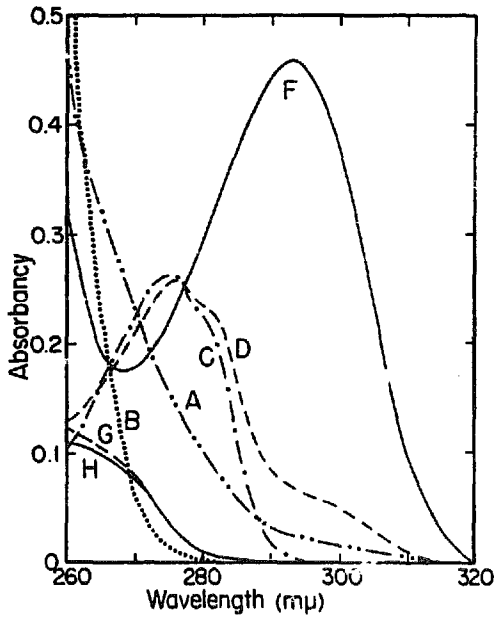

Fig. 1. Absorption spectra of CyF, cyanuric acid, tyrosine and the reaction products of CyF and tyrosine: $2.22 \cdot 10^{-2}$ M CyF in dioxane (A); $2.22 \cdot 10^{-2}$ M cyanuric acid in 10% dioxane at pH 9.1 (B); 0.196 mM tyrosine in water at pH 7.0 (C), 9.1 (D) and 12.6 (F); 0.196 mM tyrosine plus $2.22 \cdot 10^{-2}$ M CyF in 10% dioxane at pH 9.1 (G) and 12.6 (H) after 1.5 h reaction.

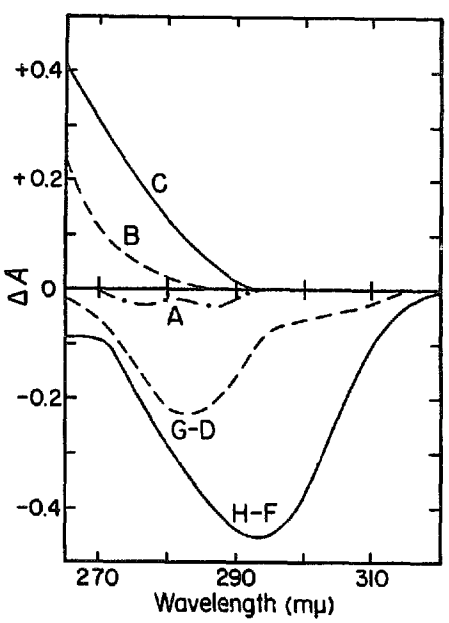

Fig. 2. Difference spectra before and after treatment of 0.196 mM amino acids with $2.22 \cdot 10^{-2}$ M CyF: tyrosine at pH 9.1 (Curve G minus Curve D in Fig. 1) and 12.6 (Curve H minus Curve F in Fig. 1); tryptophan at pH 9.1 (A); cysteine at pH 9.1 (B); histidine at pH 9.1 (C).

An appreciable spectral change was observed upon addition of CyF to tryptophan, cysteine and histidine, and practically no change on the addition to other essential amino acids. The difference spectra obtained for these three amino acids at pH 9.1 are shown by Curves A, B and C in Fig. 2. The band of tryptophan changes slightly, and the bands of cysteine and histidine are enhanced considerably but below 290 mμ, so that the changes do not affect the photometry at 295 mμ. The changes observed for these amino acids at pH 12.6 were similar to those obtained at pH 9.1.

The procedure for proteins described in the experimental part is based upon these results on amino acids, and its experimental basis may be summarized as follows. Both the product formed in the reaction of CyF with tyrosine and cyanuric acid as the by-product show no light absorption at 295 mμ, the peak in the difference spectrum of the ionization of tyrosine. Therefore, the concentration of residual and non-reacted tyrosine residues of proteins can be estimated by measuring the absorbancy difference, ΔA, at 295 mμ between the two mixtures derived from the reaction mixture by adjusting the pH value to 7.0 and 12.0–12.5, respectively. In this way, one can measure the non-reacted residue concentrations in the reaction mixtures of various pH values on the same experimental basis, and eliminate the effects of overlapping of the bands of other amino acids and their changes upon addition of CyF and alkali because other amino acids and their reaction products show no change of absorbancy at 295 mμ upon addition of alkali. Since the concentration of tyrosine residues before treatment with CyF can be measured similarly in terms of ΔA, the difference between the ΔA values before and after the treatment gives the value for the reacted tyrosine residues. To avoid confusion in the further

descriptions, the decrease of ΔA by treatment with CyF is denoted as ΔA^*, the value being proportional to the moles of the reacted tyrosine residues.

The reactivity of CyF with tyrosine was examined in this manner as a function of CyF concentration with 0.180 mM tyrosine at pH 9.1 and 12.4, and the results are shown in Fig. 3 by open and solid circles, respectively. With increasing CyF concentration, the value of ΔA^* increases and reaches the level of $\Delta A^* = 0.41$–0.42 at these pH values. This value of ΔA^* is in good agreement with the theoretical value calculated from the tyrosine concentration on the assumption that all tyrosine in the reaction mixture reacted with CyF. With $2.22 \cdot 10^{-2}$ M CyF and 0.196 mM tyrosine the pH dependence of the reactivity was examined. The result shown by solid triangles in Fig. 4 indicates that the ΔA^* value is constant between pH 9.0 and 12.7 and decreases outside these pH limits. The drop at lower pH values is, probably, due to the decrease in reactivity, and the drop at higher pH values may be an indication that the hydrolysis of CyF is more rapid than the reaction with tyrosine. Most proteins are native at pH 9.0 and are denatured around pH 12. Thus, the effect of alkali denaturation on the states of the tyrosine residues can be studied by use of this constant reactivity over the wide range of pH, as demonstrated later for insulin.

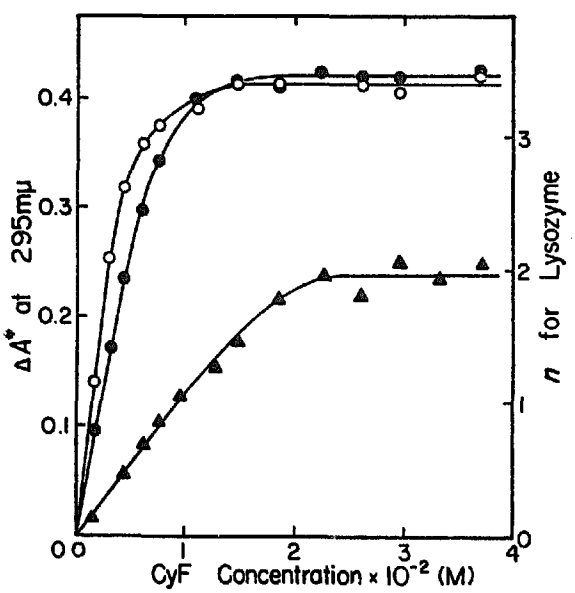

Fig. 3. ΔA^* values observed for 0.180 mM tyrosine and the tyrosine residues of 48.4 μM lysozyme as a function of CyF concentration. The scale on the right side of the figure indicates the value of n, *i.e.* the moles of tyrosine residues reacted per mole of lysozyme. O—O, tyrosine (pH 9.1); ●—●, tyrosine (pH 12.4); ▲—▲, lysozyme (pH 9.4).

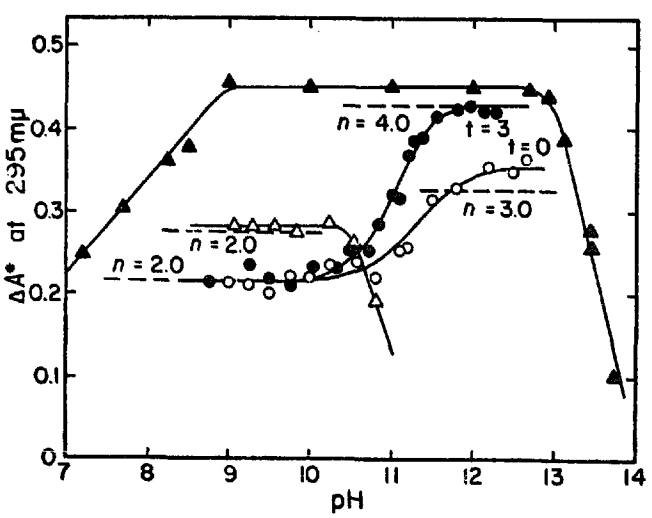

Fig. 4. The pH dependence of ΔA^* in the reactions of CyF with tyrosine, lysozyme and insulin: ▲—▲, 0.196 mM tyrosine plus $2.22 \cdot 10^{-2}$ M CyF; △—△, 55.2 μM lysozyme plus $2.96 \cdot 10^{-2}$ M CyF; 46.6 μM insulin plus $1.85 \cdot 10^{-2}$ M CyF at $t = 0$ (O—O) and $t = 3$ h (●—●).

(2) Tyrosine residues of lysozyme

In presence of 4 M guanidine, all of the tyrosine residues of lysozyme ionize rapidly with alkali[5]. Curve A in Fig. 5 is an example of the difference spectrum of the ionization observed with 48.4 μM lysozyme, and shows a peak at 298 mμ, the wavelength being slightly longer than 295 mμ, observed for tyrosine and insulin. By treatment with $2.59 \cdot 10^{-2}$ M CyF at pH 9.7, the peak in the difference spectrum is lowered to approximately one-third the original height (Curve B). The drop, ΔA^*, of the height was observed as a function of CyF concentration at the same pH and lysozyme concentration. The result shown in Fig. 3 indicates that the minimum CyF concentration for completion of the reaction is $2.5 \cdot 10^{-2}$ M. Open triangles in Fig. 4 show the pH dependence of ΔA^* observed with $2.96 \cdot 10^{-2}$ M CyF and 55 μM lysozyme, and indicate that the ΔA^* value is constant below pH 10.3 but drops above this pH. The drop of ΔA^* is probably due to the precipitates formed above the same pH value. The dashed horizontal line near the curve shows the level of $n = 2.0$ calculated from the lysozyme concentration, and is close to the observed level of ΔA. It is evident from this result that two of the total three residues in the lysozyme molecule are reactive with CyF and the remaining one is of the non-reactive type.

The question may arise as to whether or not this non-reactive residue is identical with the slowly ionizing residue identified by INADA[5]. To answer this question, a reaction mixture of pH 9.3 was made alkaline without addition of guanidine. The ionization of the residual residue proceeded slowly, and it took several hours to obtain a constant reading of ΔA corresponding to $n = 1.0$. This fact bears evidence that the non-reactive residue is identical with the slowly ionizing residue of INADA.

(3) Tyrosine residues of insulin

The reaction of CyF with insulin could be observed without precipitation over the full pH range of the constant reactivity of CyF. The difference spectrum (Curve C in Fig. 5) between the neutral and alkaline solutions of insulin showed a positive peak at 295 mμ, as previously observed[5]. The ΔA value at this wavelength drops on treatment with CyF. The drop, ΔA^*, with an excess of CyF is dependent on the pH value of the reaction mixture and, at higher pH values, also dependent on the time of alkali denaturation; *i.e.*, the time during which the alkaline insulin solution was left standing at room temperature before addition of CyF. Curve D in Fig. 5 shows an example of the difference spectrum in an extreme case when insulin was denatured at pH 12.5 for 3 h and then treated with $1.85 \cdot 10^{-2}$ M CyF at the same pH. The ΔA value after the treatment is negligibly small, indicating that all of the tyrosine residues reacted with CyF. The dependence of ΔA^* on CyF concentration was observed at three different conditions; pH 12.4 and t (the denaturation time in h) = 0 and 3; and pH 9.0 and $t = 0$. The minimum CyF concentration for completion of the reaction thus determined was $1.5 \cdot 10^{-2}$ M, being the same under these different experimental conditions (Fig. 6).

With 46.6 μM insulin and $1.85 \cdot 10^{-2}$ M CyF, the pH dependence of ΔA^* was observed at $t = 0$ and 3. The pH value of a reaction mixture in the measurements was the same as the value for the sample insulin solution before addition of CyF. Between pH 9.0 and 10.0, the ΔA^* values for the different incubation times are identical with each other and are constant on the level of $n = 2.0$, as seen from the data shown in Fig. 4. This indicates that two of the total four tyrosine residues in the native insulin

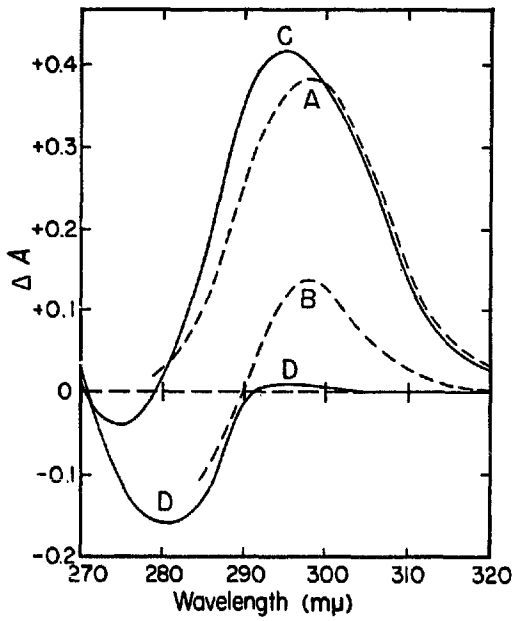

Fig. 5. Changes in the difference spectra of ionization of the tyrosine residues of lysozyme (dashed curves) and insulin (solid curves) by treatment with CyF: 48.4 μM lysozyme before treatment (A) and after treatment (B) with $2.59 \cdot 10^{-2}$ M CyF at pH 9.7; 46.6 μM insulin before treatment (C) and after treatment (D) with $1.85 \cdot 10^{-2}$ M CyF at pH 12.5. The insulin sample solution was kept at the alkaline pH for 3 h before treatment with CyF.

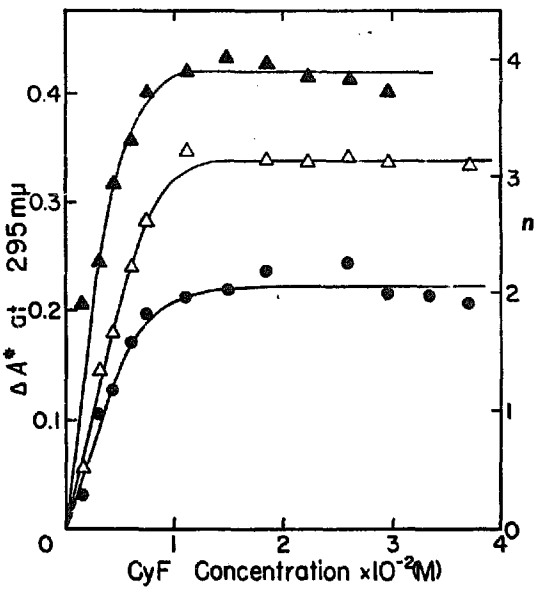

Fig. 6. Changes of ΔA^* observed for 46.6 μM insulin as a function of CyF concentration at three different conditions of pH and t; ▲—▲, $t = 3$ at pH 12.4; △—△, $t = 0$ at pH 12.4; ●—●, $t = 0$ at pH 9.0. The scale on the right side of the figure indicates the moles, n, of the tyrosine residues reacted per mole of insulin.

molecule are of the CyF-reactive type, so that the remaining two are of the non-reactive type. On increasing the pH, the ΔA^* value for the different incubation times increases and reaches different levels of $n = 3.3$ and 4.0. The latter value indicates that the two non-reactive residues were transformed into the reactive type during 3 h of alkali denaturation. The former value, on the other hand, suggests that there exists a difference in the transformation rate between the two non-reactive residues. According to INADA[5], exactly three residues ionize instantaneously upon addition of alkali and the remaining one residue slowly. Considering this fact, one of the two non-reactive residues may have been transformed into the reactive type immediately after preparation of the alkaline solution of insulin. The time, 3 h, required for the ionization of the slowly ionizing residue agrees with the time for the complete transformation into the reactive type. The deviation of the former value from 3.0 may, therefore, be explained as follows. It takes roughly 30 min for the hydrolysis of CyF, and the slower transformation may proceed partially before the complete hydrolysis even when CyF is added immediately after preparation of the alkaline sample solution. Hence, some of the residues transformed after addition of CyF would react with CyF, causing the deviation. The pK value for the transformation of the two non-reactive residues was estimated to be 11.0, and the order of the sigmoid transformation curve was found to be 2.0 ± 0.1. This fact implies that two hydroxyl ions are involved in the transformation of each of the two non-reactive residues. This contrasts with the fact that one hydroxyl ion is involved in the ionization of the slowly ionizing residue[5].

The distribution of the two non-reactive residues in the A and B chains of insulin was determined in the following manner. Native insulin was first treated with CyF at pH 9.3. The protein in the reaction mixture was precipitated with 1.0 M trichloroacetic acid, and washed several times with 1.0 M trichloroacetic acid solution. The disulfide bonds of this CyF-treated insulin were cleaved with performic acid, and the two peptide chains were separately obtained by the method of KOTAKI[22]. The yields of the A and B chains were 70 and 81 %, respectively. The A chain was dissolved in bicarbonate buffers of pH 7.0 and 12.0, and the B chain was dissolved in the same buffers but with the aid of 4 M guanidine, because it was insoluble in the buffers without guanidine. The number, n, of the residual tyrosine residues in the A and B chains was estimated from the ΔA values for these solutions, and was found to be 1.08 and 1.03, respectively. It may be concluded from this result that there is one non-reactive residue in each of the two chains.

DISCUSSION

Let us first compare the numbers of CyF-non-reactive residues in the native lysozyme and insulin molecules with those identified by INADA[5] based on the rate of ionization. One of the three residues in the lysozyme molecule is of the non-reactive type, and this residue was proved to be identical with the slowly ionizing residue. Two residues of the non-reactive type were identified in the native insulin molecule, and one of them was found to be identical with the slowly ionizing residue. The four tyrosine residues may, therefore, be classified into three groups: two CyF-reactive and rapidly ionizing residues, one CyF-non-reactive but rapidly ionizing residue, and one CyF non-reactive and slowly ionizing residue.

As indicated above, two bound residues were identified by the group of SCHERAGA[9-12] based upon the spectral changes on tryptic digestion and on acidifying the solutions of both native insulin and its tryptic digest. This number of bound residues agrees with the number determined with CyF. However, the agreement in number does not necessarily imply identity. In fact, one of the two bound residues of SCHERAGA *et al.* is the B26 residue and the other was inferred by them to be the B16 residue, while of the two CyF-non-reactive residues one is in each of the A and B chains. Therefore, if the inference on the B16 residue is true, there must be three bound residues in the insulin molecule. In connection with this interpretation, the reactivities of the four residues with iodine compared by DE ZOETEN *et al.*[13,14] are of great interest. The less reactive nature of the two residues in the B chain accords with the conclusion drawn by SCHERAGA *et al.* The A14 residue was normal, being iodinated into its diiodo derivative with the rate similar to that observed for tyrosine, while the A19 residue was abnormal in that it takes up only one iodine atom per residue in the same experimental conditions and that the reactivity at low degrees of iodination is greater than that of tyrosine. If this abnormality is reflected in the reactivity with CyF, the CyF-non-reactive residue in the A chain would be the A19 residue rather than A14. The question as to the assignment of the two CyF-non-reactive residues may be answered by spectroscopic measurements of peptides derived from the CyF-treated insulin by digestion with various proteinases. The results of these measurements will be reported in a future paper.

The application of CyF to lysozyme and insulin differentiated their tyrosine

residues distinctly into two types. However, what state(s) of tyrosine residues is essential for the marked difference in the reactivity is obscure. The reactivity is inferred to be suppressed by hydrogen bonding. However, a tyrosine residue embedded in the interior of a protein molecule may not react with CyF, and a steric hindrance may also affect the reactivity. It is clear that the slowly ionizing residues of both lysozyme and insulin do not react with CyF. This indicates that there exists a bound state reflected in these different properties.

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