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Location of Abnormal Tyrosines in Actin*

Koshin Mihashi and Tatsuo Ooi

ABSTRACT: Spectrophotometric titrations on G- and F-actin indicate that almost all the tyrosines are buried inside the molecule. Correlated with exposure of these buried tyrosines at high *p*H, an irreversible denaturation, or loss of polymerizability, took place. All the tyrosine residues were titrated at *p*H higher than 12 and were reabnormalized by lowering the *p*H, indicating that a refolding of the molecule occurred and masked some of the tyrosines. Guanidine HCl normalized the abnormal tyrosines, since a reversible titration curve with the normal *pK* of phenolic groups was obtained in 5 M guanidine HCl solution. In this solution, splitting of a monomer into two subunits occurs according to Adelstein and co-workers (R. S. Adelstein, J. E. Godfrey, and W. W. Kielley, *Biochem. Biophys. Res. Commun. 12*, 34 [1963]). When guanidine HCl was dialyzed out against water containing 30 mM KOH, the subunits did not recombine to form a monomer or some aggregate, as judged from the molecular weight (Archibald method) and the sedimentation constant obtained for this solution.

The effect of increasing the concentration of guanidine HCl added to actin solution at *p*H 10 demonstrated two steps in the normalization of the tyrosines, suggesting the existence of two kinds of abnormal groups. Another denaturing reagent, urea, could not normalize all the tyrosines, indicating that the action of urea is different from that of guanidine HCl. The results suggest that the molecule has a rigid core with deeply buried tyrosines.

Actin, one of the muscle proteins (Straub and Feuer, 1950), exists in a globular form (G-actin) of a molecular weight of about 6×10^4 in salt-free solutions (Mommaerts, 1952; Lewis *et al.*, 1963; Mihashi, 1964). On addition of neutral salts (e.g., KCl), G-actin polymerizes into a long fibrous molecule (F-actin) (Straub and Feuer, 1950). Electron-microscopic studies of F-actin by Huxley (1963) and by Hanson and Lowy (1963) show that G-actin molecules are arranged to form the double-stranded helical structure of F-actin, so that each G-actin molecule seems to interact with four neighbors by an attractive force of specific importance for the polymerization (Oosawa and Kasai, 1962; Asakura *et al.*, 1963). However, there are few reports on the structure of actin which elucidate the molecular mechanism of its polymerization (Higashi *et al.*, 1963).

Recently, Higashi and Oosawa have found a significant difference spectrum between G-actin and F-actin (S. Higashi and F. Oosawa, to be published), suggesting an increase in the content of α helix and/or β structure of the molecule associated with polymerization. Another important result has been obtained by Adelstein *et al.* (1963), who showed that a monomer of actin is composed of two subunits of similar, if not identical, mass; a value of 28,200 for the average molecular weight of these subunits has been determined by short-column sedimentation equilibrium in 5 M guanidine hydrochloride solution.

It is therefore of great importance to obtain further knowledge of the structure of the actin molecule, including side-chain interactions, by carrying out physicochemical measurements on actin solutions. With these results we expect to clarify the relationship between the structure and function (i.e., polymerizability) of actin.

* From the Department of Physics, Faculty of Science, Nagoya University, Nagoya, Japan. *Received November 24, 1964; revised January 25, 1965.*

Experimental

Material. Crude actin was extracted from acetone-dried rabbit skeletal muscle (Straub and Feuer, 1950) with freshly distilled water at 0° (Martonosi, 1962). Purification was carried out according to the following procedure (Mihashi, 1964). The crude extract was partly polymerized by the addition of 30 mM KCl, stirring gently for *ca.* 30 minutes at room temperature. Then sonic vibratton was applied to a portion of the partially polymerized solution for 1 minute, resulting in complete polymerization. Polymerization of the rest of the solution was accelerated by mixing in of the sonicated solution (Asakura *et al.*, 1963), a procedure which increased the yield of F-actin. The F-actin was spun down by centrifugation, using a Spinco Model L ultracentrifuge at 3.5×10^4 rpm for 150 minutes, dispersed into 60 mM KCl–0.5 mM ATP[1] solution with stirring and sonication, and spun down again. This washing process was repeated twice. Impurities such as tropomyosin can be removed by this procedure (Mihashi, 1964). Finally, the F-actin pellet was dispersed into cold water containing 0.2 mM ATP, at neutral *p*H. After dialysis against water containing 10^{-4} M sodium carbonate and 2×10^{-5} M ATP, the solution was centrifuged at 3.5×10^4 rpm for 30 minutes to remove aggregates. The concentration of the original G-actin solution thus purified was usually 5–7 mg/ml.

Guanidine hydrochloride and urea, of reagent grade, were obtained from Katayama Chemical Industry Co. Other chemicals were analytical grade where possible. Protein concentrations were determined by biuret reaction after calibration as described in the previous paper (Mihashi, 1964).

Difference Spectrum and Spectrophotometric Titration. A Zeiss PMQ II spectrophotometer was used to measure ultraviolet difference spectra and spectrophotometric titrations in 10-mm quartz cells, using a neutral solution of the same protein concentration as a reference (Laskowski *et al.*, 1956). Titration measurements were carried out mainly at 295 mμ.

Titration measurements were made with a Radiometer *p*H-stat (TTTl), titrating forward by adding 3 M KOH and backward by adding 3 M HCl. The protein concentration was 0.4–0.7 mg/ml. Correction was made for dilution by the base or acid added.

Water from a temperature-controlled bath at 20° was circulated through the thermospacer of the spectrophotometer and a jacket of the titration cuvet of the *p*H-stat.

Ultracentrifuge Measurements. The determination of molecular weight was performed by the Archibald method using standard procedure (Archibald, 1947; Schachman, 1959). All runs were made at 20,410 rpm at 7° in conventional 12-mm cells with 4° sector Kel-F centerpieces. Photographs were taken with schlieren phase plate at a bar angle of 70°, and more than five frames were recorded in each run. The molecular weight was calculated only from measurements on the meniscus. The partial specific volume of guanidine HCl–treated actin, after dialysis to remove guanidine HCl against water, was assumed to be 0.732 ml/g, the value obtained for G-ADP actin in water (Mihashi, 1964).

Sedimentation constants were calculated from measurements at 59,780 rpm. Measurements were carried out at 11.5° with an RTIC temperature control device.

Optical Rotatory Dispersion. Measurements were carried out by a Rudolph photoelectric polarimeter, Model 80, equipped with a Beckman DU quartz monochrometer. Light from a General Electric high-pressure mercury arc, AH-6, was used at wavelengths of 366, 405, 436, 546, and 579 mμ. A jacketed polarimeter cell of 5-mm bore with quartz end plates was employed. Temperature was maintained at 20°.

Optical rotatory dispersion data were plotted according to the Moffit-Yang equation (Moffit and Yang, 1956):

$$[m'] = \frac{a_o\lambda_o^2}{\lambda^2 - \lambda_o^2} + \frac{b_o\lambda_o^4}{(\lambda^2 - \lambda_o^2)^2}$$

where $[m']$ is the reduced mean residue rotation, and the value of λ_o was taken as 212 mμ in order to evaluate the dispersion parameters, a_o and b_o.

Polymerizability was measured by a flow birefringence determination of the amount of F-actin which polymerized through the addition of salts to a G-actin solution (Oosawa *et al.*, 1959). The conditions used for polymerization were 0.06 M KCl, 0.005 M Tris-HCl buffer, *p*H 8.0.

Treatment with guanidine HCl *and urea* was done by incubation usually for a day or longer at 0°, in order to avoid time-dependent effects. Dialysis of guanidine HCl was carried out against a large volume of water containing 30 mM KOH, at 0° for 3 days.

Results

Difference Spectrum of Actin. It has been reported (Hermans, 1963; Donovan, 1964) that the difference spectrum for ionization of a tyrosine residue has two maxima, one at 245 mμ and the other at 295 mμ, the extinction coefficient of the former being about five times as large as that of the latter. As shown in Figure 1, the difference spectrum for actin at *p*H higher than 10, referred to a solution of the same protein concentration at neutral *p*H, had two maxima at 245 mμ and at 295 mμ, and the ratio of these two maxima was 4.9. The heights of the peaks increased with *p*H, apparently owing to ionization of phenolic groups in the molecule. Since no shoulder was observed near 290 mμ, which might arise from tryptophan chromophores (Bigelow and Geschwind, 1960), the difference spectrum measures only ionization of tyrosine groups usually observed in a spectrophotometric titration at a wavelength of 295 mμ (Beaven and Holiday, 1952; Tanford and Hauenstein, 1956). Strong support for this result

[1] Abbreviations used in this work: ATP, adenosine-5′-triphosphate; ADP, adenosine-5′-diphosphate.

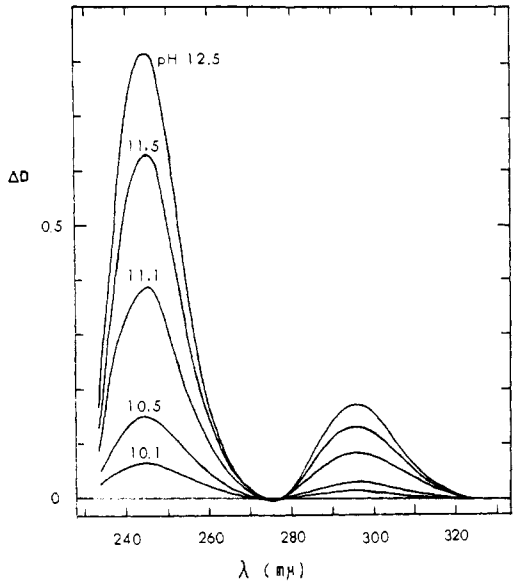

FIGURE 1: The difference spectra of actin as a function of *p*H. Protein concentration is 0.17 mg/ml. The reference solution is of the same protein concentration at *p*H 7.3.

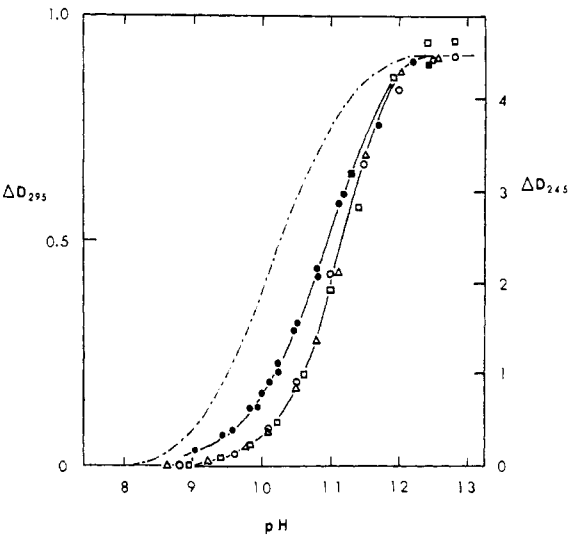

FIGURE 2: Spectrophotometric titration curves of G-actin and F-actin referred to the same solution at neutral *p*H. G-Actin: ○, forward at 295 mμ, C_p = 0.65 mg/ml; △, forward at 245 mμ, C_p = 0.17 mg/ml; ●, reverse from *p*H 12.8 at 295 mμ, C_p = 0.65 mg/ml. F-Actin in 60 mM KCl: □, forward at 295 mμ, C_p = 0.67 mg/ml; ■, reverse from *p*H 12.8 at 295 mμ, C_p = 0.67 mg/ml. The ordinate is the optical density at 295 mμ, ΔD_{295}, per gram of protein per liter. The dashed line represents the titration curve of actin in 5 M guanidine HCl solution (Figure 4).

was found in agreement of titration curves measured at 245 and 295 mμ over the *p*H range of the experiment (Hermans, 1963) (see Figure 2). Although it is conceivable that contribution of chromophores other than tyrosine to the absorption spectrum might be present, such a contribution seems to be very small, presumably because of adequate choice of the reference solution.

In the experiments described below, including those for guanidine HCl and urea solutions, the difference spectrum was used to demonstrate that spectrophotometric titration at 295 mμ corresponded to titration only of tyrosines. All results showed no contribution from tryptophan residues.

The spectrophotometric titration curve for G-actin is shown in Figure 2. A gradual increase with *p*H in the difference spectrum at 295 mμ, ΔD_{295}, began around *p*H 9.5, followed by a sharp increase at about *p*H 10.7. An apparent *pK*, the *p*H where ΔD_{295} gives a half value of the maximum, ΔD_{295}^{max}, was estimated to be 11.1 from the curve. This apparent *pK* is higher than the normal *pK* of tyrosine, usually about 10.0 in the presence of salts (Donovan *et al.*, 1959). Taking into account the electrostatic effect on the titration, the apparent *pK* is shifted from the intrinsic *pK* 9.6 to a higher value dependent on salt concentration (Donovan *et al.*, 1959), which does not exceed *p*H 10.4. Therefore the curve indicates that most of tyrosine residues are buried in the interior of the molecule.

The total number of tyrosine residues is 24 per molecular weight of 6×10^4, calculated from ΔD_{295}^{max} at *p*H 13.5 by assuming the molar extinction at 295 mμ of an ionized phenolic group as 2300 (Beavan and Holiday, 1952). This number is in agreement with that obtained from amino acid analysis, i.e., 19 residues (Kominz *et al.*, 1954) and 22 residues (Carstein, 1963), within experimental error.

A similar titration curve was obtained for F-actin in solution, as shown in Figure 2. The data fall on the titration curve of G-actin, showing that there is no apparent difference between titrations of G- and F-actin. From this curve it is evident that most tyrosine chromophores are buried inside the molecule as they were in G-actin, and that all the tyrosines are titratable. We cannot exclude the possibility that the tyrosines of F-actin might have a little lower *pK* than those of G-actin, taking into account electrostatic effects (Scheraga, 1961; Donovan *et al.*, 1959), although the titration curve coincided with that of G-actin.

When back titration was carried out on G- and F-actin, both sets of data lay on the same curve, as shown in Figure 2. The apparent *pK* was estimated as 10.7, still higher than the normal *pK* of tyrosine. The actin once exposed at high *p*H has no more polymerizability. Since all tyrosines are ionizable at *p*H 13.5, the result suggests that refolding of the molecule occurs in such a way that some of tyrosine groups are buried again inside the molecule, taking presumably a conformation similar to but different from the native. The forward titration curve of this denatured actin was between those of the forward and backward titration of native actin, whereas back titration of this material once more traced the same curve as for G- and F-

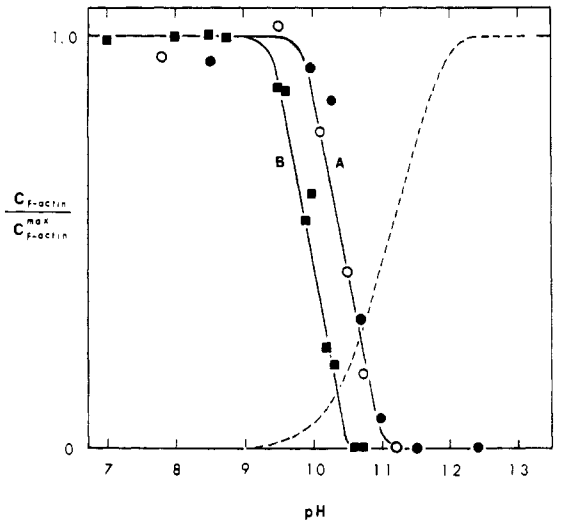

FIGURE 3: The *p*H dependence of the denaturation of G- and F-actin, and of the depolymerization of F-actin. The ordinate is the ratio of concentration of F-actin to maximum concentration of F-actin determined by the flow birefringence method. Total concentration of F-actin is about 1.5–2.0 mg/ml. Curve A: O, denaturation of G-actin; after raising the *p*H of the G-actin solution to the values indicated, the G-actin was polymerized by the addition of Tris-HCl buffer and KCl (final concentrations, Tris-HCl 10 mM, KCl 60 mM). ●, F-actin; after raising the *p*H of F-actin solutions to the values indicated, Tris-HCl buffer (*p*H 8.0) of a final concentration of 10 mM was added. Curve B: depolymerization of F-actin; measurements of flow birefringence were carried out at the raised *p*H. The dashed line represents the results of spectrophotometric titration of G- and F-actin at 295 mμ in arbitrary units (see Figure 2).

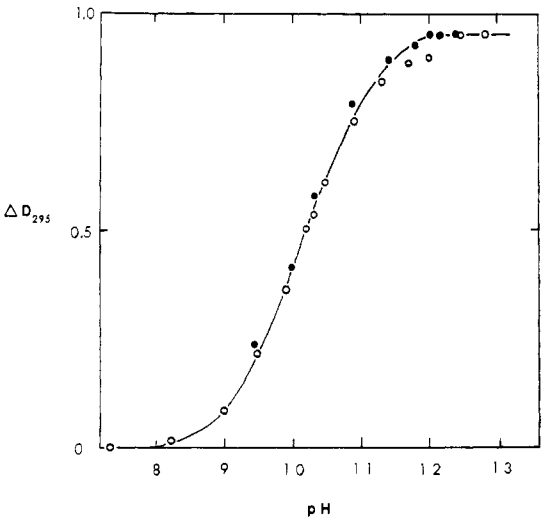

FIGURE 4: Spectrophotometric titration curve of actin in 5 M guanidine HCl referred to the solution of the same concentration of protein at neutral *p*H. Protein concentration is 0.34 mg/ml. The ordinate is the optical density at 295 mμ, ΔD_{295}, per gram of protein per liter. O, forward at 295 mμ; ●, reverse from *p*H 12.4 at 295 mμ.

actin, expected from the denaturation caused by exposure at high *p*H.

To examine the correlation between polymerizability (reflecting depolymerization and denaturation) and ionization of the tyrosine residues, polymerizability of G-actin at *p*H 8.0 was plotted against the *p*H to which G-actin solutions had been raised, as shown in Figure 3. Similar data for F-actin solutions are presented in the same figure. The results follow a single curve (curve A), indicating that there is no difference in denaturation caused by *p*H between G-actin and F-actin, i.e., denaturation in the absence or the presence of salts. Apparently actin sharply loses its polymerizability at around *p*H 10.5. The degree of polymerization of F-actin versus *p*H is also shown in Figure 3 (curve B). Depolymerization of F-actin occurred in parallel with the denaturation described, but the *p*H of a sharp decrease in polymerization, 10.0, was about 0.5 *p*H unit lower than that of denaturation, 10.5.

The *p*H dependence of a reversible G-F transformation is obtained by the ratio of the degree of polymerization at a given *p*H to that of survived native actin which polymerized at neutral *p*H. The ratio can be estimated from the depolymerization curve (B) and the denaturation curve (A) in Figure 3. The curve thus calculated was almost the same as for the *p*H dependence of the degree of polymerization (B), indicating that the transition of F form to G form occurs at about *p*H 10.0. At this *p*H a few tyrosines are ionized, as shown in the broken line in the figure. Although it is not possible to conclude whether or not tyrosine groups are involved in the polymerization process, it is quite likely that some charged groups having a *pK* of about 10 play an important role in the G-F transformation. It is worthwhile to note that the depolymerization of F-actin with raising of the *p*H occurs quickly, in contrast to the slow depolymerization induced by lowering the salt concentration (Straub and Feuer, 1950).

Treatment with 5 M Guanidine Hydrochloride. The titration curve of actin in 5 M guanidine HCl is shown in Figure 4. The titration was reversible and the apparent *pK* obtained from this curve was 10.1 (Cha and Scheraga, 1960). Since 5 M guanidine HCl shifts the *pK* for a phenolic group about 0.5 *p*H unit higher than the intrinsic *pK* of 9.6 for a normal phenolic group in water (Donovan *et al.*, 1959), the normal ionization of tyrosine groups in 5 M guanidine HCl is expected to occur with a *pK* of 10.1. The total number of tyrosines ionized at *p*H 13, estimated by the use of a molar extinction of 2300 (Beaven and Holiday, 1952), is in agreement with that described before. Therefore all the tyrosines are titrated normally, i.e., the buried tyrosines are exposed to solvent by the addition of 5 M guanidine HCl. According to the results of Adelstein

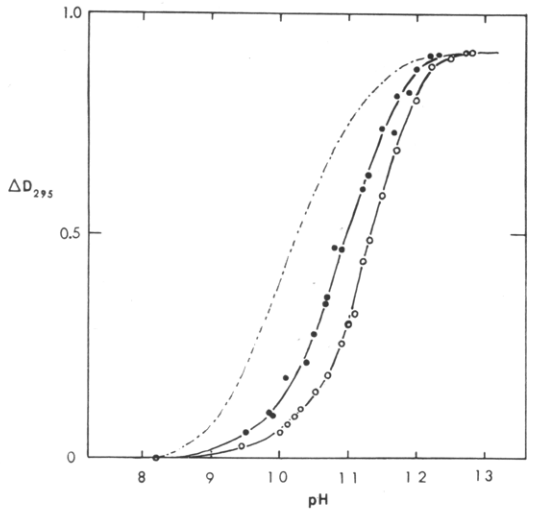

FIGURE 5: Spectrophotometric titration curves of 5 M guanidine HCl–treated actin after dialyzing away the guanidine HCl, referred to the same solution at neutral *p*H. Protein concentration is 0.35 mg/ml. The ordinate is the optical density at 295 mμ, ΔD_{295}, per gram of protein per liter. ○, forward; ●, backward from *p*H 12.4. The dashed line represents the result of spectrophotometric titration in 5 M guanidine HCl (Figure 4).

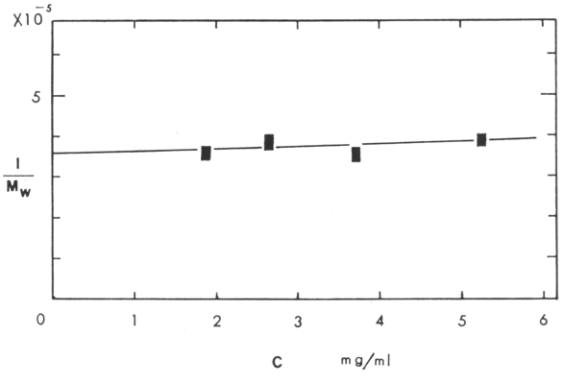

FIGURE 6: Concentration dependence of the reciprocal of the apparent molecular weight of 5 M guanidine HCl–treated actin, after dialyzing away the guanidine HCl. Archibald method at 7°. Actin was incubated in 5 M guanidine HCl overnight, then the guanidine salt was dialyzed from cold solution which contained 30 mM KOH and 1 mM cysteine.

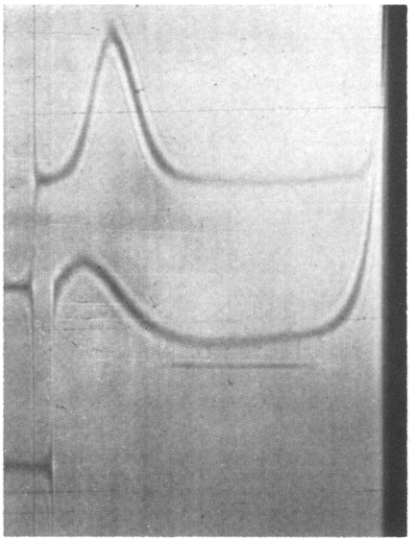

FIGURE 7: Sedimentation patterns of G-actin (upper) and of 5 M guanidine HCl–treated actin after dialyzing away the guanidine HCl (lower). Protein concentration 5.6 mg/ml, KOH 30 mM, 5.9°; bar angle 55°, rotor speed 59,780 rpm, 137 minutes after reaching speed; sedimentation direction is from left to right.

et al. (1963), dissociation into half-molecules of an actin monomer takes place in 5 M guanidine HCl, suggesting unfolding of the molecule. The reversible titration, which was not observed for G- or F-actin, suggests that an unfolded conformation is present in neutral solutions of guanidine HCl, as suggested by the absence of helix in this medium (Nagy and Jencks, 1962).

The titration curve in Figure 5, which was obtained on the solution after removing guanidine HCl by dialysis against water containing 30 mM KOH, showed reabnormalization of the tyrosine groups. The apparent *pK* of this curve was almost the same or a little higher than that of G- or F-actin, and the back titration curve was coincident with the curve for the native proteins. This result indicates that refolding occurs so as to bury tyrosine groups inside the molecule.

A molecular weight of actin in 30 mM KOH after guanidine HCl was dialyzed out was determined by means of the Archibald technique. As shown in Figure 6, a value of 29,500 was obtained after extrapolation to infinite dilution, indicating that subunits obtained in 5 M guanidine HCl (Adelstein *et al.*, 1963) exist separately even after removal of guanidine HCl, and do not recombine with each other. Sedimentation patterns of the subunit after removal of guanidine HCl and of G-actin are shown in Figure 7; both patterns show single peaks of monodisperse shape, indicating that there is no aggregate in these solutions. The sedimentation constant of the subunit calculated from the position of the peak in the figure is 1.38 S, half that of G-actin, 2.79 S. This result again indicates the presence of a subunit, since the two measurements were carried out under the same conditions, the only difference being with or without pretreatment with guanidine HCl. Figure 8 shows the concentration dependence of the sedimentation constant of the subunit after removal of guanidine HCl. A value of 1.73 S was obtained at infinite dilution.

Furthermore, the subunit after removal of guanidine HCl had no helix, a conclusion inferred from the measurement of optical rotatory dispersion at *p*H 12.5 and

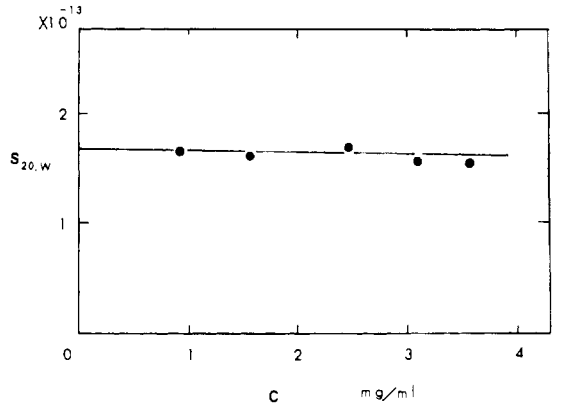

FIGURE 8: Concentration dependence of the sedimentation rate of 5 M guanidine HCl-treated actin. In 30 mM KOH, at 11.5°.

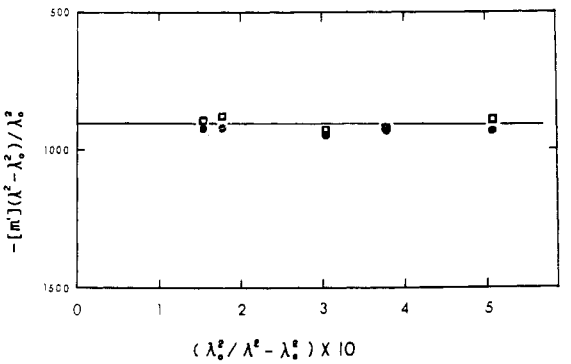

FIGURE 9: Moffit plots for the optical rotatory dispersion of 5 M guanidine HCl-treated actin after dialyzing away guanidine HCl. ●, *p*H 12.5 (KOH 30 mM), protein concentration 12.4 mg/ml. □, *p*H 10.2 (glycine buffer 25 mM), protein concentration 4.9 mg/ml.

10.2, shown in Figure 9. Plots of $[m'](\lambda^2 - \lambda_0^2)/\lambda_0^2$ versus $\lambda_0^2/(\lambda^2 - \lambda_0^2)$ were parallel to the abscissa, so that the value of b_0 was zero. Since the existence of abnormal tyrosines at *p*H 10.2 is obvious from the curve in Figure 5, it is concluded that normalization of tyrosines in 5 M guanidine HCl is not caused by dissociation into half-molecules but by unfolding of the molecule, and that some refolding must occur after removal of guanidine HCl, giving rise to abnormal behavior of tyrosine groups. Aggregates of subunits were found in sedimentation analysis of a solution which had been kept standing after the *p*H of the dialyzed solution had been lowered to neutrality, a result which suggests that the subunits refold so as to enhance a small attractive force between molecules. Buried tyrosines may play a role in the stabilization of such a refolded conformation (Hermans and Scheraga, 1961), inferred from their abnormal behavior. The addition of cysteine to guanidine HCl solutions produced no effect, suggesting that

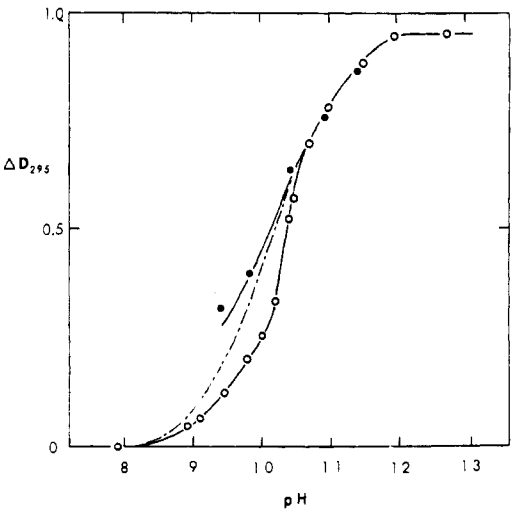

FIGURE 10: Spectrophotometric titration curves of actin in 2 M guanidine HCl solution referred to the same solution at neutral *p*H. Protein concentration is 0.34 mg/ml. The ordinate is the optical density at 295 mμ, ΔD_{295}, per gram of protein per liter. ○, forward. ●, reverse from *p*H 12.8. On the forward titration curve, a transition is undergone at *p*H about 10. The dashed line represents the result of spectrophotometric titration in 5 M guanidine HCl (Figure 4).

thiol groups in the molecule are not concerned in the results described above.

Stepwise Normalization of Tyrosines by Guanidine HCl. The titration curve in 2 M guanidine HCl solution shown in Figure 10 is quite different from that in 5 M solution, i.e., abnormality was observed in this solvent. Below *p*H 10 the degree of ionization was less than for normal groups but greater than for G- or F-actin. Above *p*H 10 a sharp increase occurred until at *p*H 10.5 it joined with the normal titration curve in 5 M guanidine HCl solution. This phenomenon suggests that an increase in *p*H near *p*H 10 gives rise to a transition to a looser conformation. When the solution was back titrated after the transition had occurred, a slight turbidity gradually appeared from about *p*H 10 with lowering of *p*H, and the curve seemed to coincide with the normal one in 5 M guanidine HCl after correction of the turbidity. This result shows that the transition was irreversible. Therefore abnormal tyrosines buried in 2 M guanidine HCl solution below *p*H 10 become exposed by raising the *p*H, presumably accompanying a significant irreversible change in conformation.

Since 5 M guanidine HCl normalizes the abnormal tyrosines, a ratio of ΔD_{295} in various concentrations of guanidine HCl to that in 5 M (ΔD_{295}^{max}) at a given *p*H corresponds to the fraction of the tyrosines normalized. Of course, this calculated fraction is an apparent percentage, when taking a solvent effect into account. As shown in Figure 11, approximately 65% of the tyrosines are exposed at *p*H 10 by the addition of

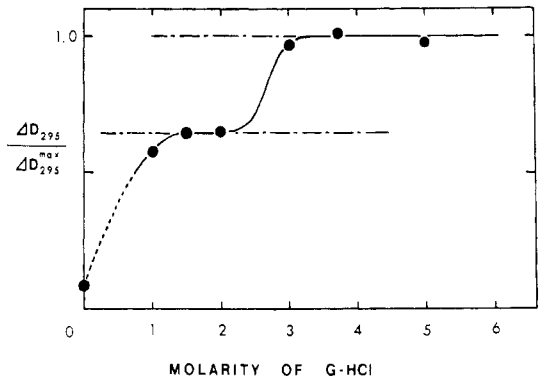

FIGURE 11: Plots of $\Delta D_{295}/\Delta D_{295}^{max}$ at *p*H 10.0 versus the concentration of guanidine HCl. The reference solution is of the same protein concentration, without guanidine HCl and at neutral *p*H. The protein concentration was 0.55 mg/ml. The dashed line represents a range of guanidine salt concentrations where actin polymerizes and precipitates (see text).

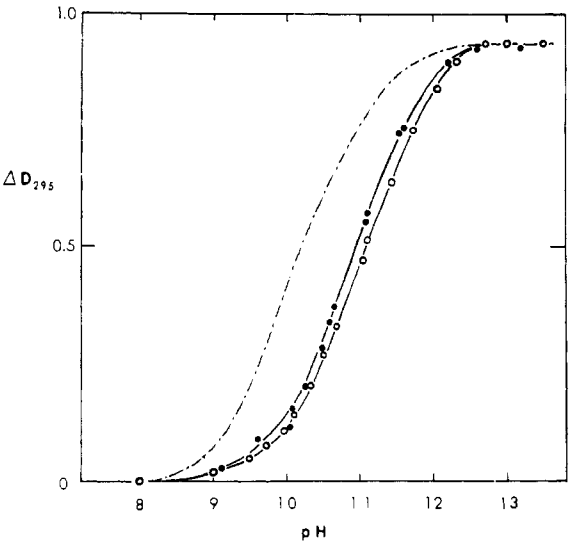

FIGURE 12: Spectrophotometric titration of actin in 6 M urea referred to the same solution at neutral *p*H. Protein concentration 0.61 mg/ml. The ordinate is the optical density at 295 mμ, ΔD_{295}, per gram of protein per liter. ○, forward; ●, reverse from *p*H 13.7. The dashed line represents the results of spectrophotometric titration of actin in 5 M guanidine HCl solution.

guanidine HCl below a concentration of 2.5 M; above this concentration a sharp increase in the ratio takes place to complete normalization of the abnormal tyrosines.

The abnormal tyrosines, therefore, can be divided into two classes; one is normalized by adding less than 2.5 M guanidine HCl and the other by further addition of guanidine HCl, the latter being more strongly abnormal than the former. The strongly abnormal tyrosines probably correspond to those which normalized at *p*H above 10 in 2 M guanidine HCl (Figure 10), since the plots of the fraction of tyrosines normalized versus *p*H estimated from the data in Figure 10 give a transition curve similar to that in Figure 11.

It is worthwhile to note that the addition of a small amount of guanidine HCl (of the order of 100 mM) to G-actin gave rise to polymerization, further addition produced precipitation, and finally the precipitate dissolved at about 1 M guanidine HCl. This region is represented by a dashed line in Figure 11.

Effect of Urea. It is well known that, in 6 M urea, actin loses its polymerizability and helical parts of the molecule are destroyed (Nagy and Jencks, 1962). However, spectrophotometric titration of actin in 6 M urea shows the existence of abnormal tyrosines, as indicated by the curve in Figure 12. The apparent *pK* was a little lower than that of G- or F-actin, but higher than that in 5 M guanidine HCl. This result indicates that part of the abnormal tyrosines become accessible to the solvent. In other words, denaturation caused by urea is not so extensive as that caused by guanidine HCl, and a folded region associated with abnormality of the phenolic groups remains in the molecule. There is no helix in the residual folded region, the optical rotatory dispersion of actin in urea solutions indicating its absence (Nagy and Jencks, 1962).

Interestingly, back titration in this solution is coincident with that of G- and F-actin, after correction for small *pK* shifts caused by urea and by KCl (Donovan *et al.*, 1959), suggesting that the denaturation or the unfolded conformation is similar in both cases. The molecule refolds with lowering of the *p*H to enhance abnormalization of tyrosine groups even in urea solution. It is concluded, therefore, that the denaturing effect of urea is not so strong as to normalize all the abnormal tyrosines in the molecule, even though helical parts of the structure are broken.

It is noteworthy that the addition of a small amount of urea to a G-actin solution did not produce any polymerization or precipitation, in contrast to the case of the addition of guanidine HCl.

In order to clarify the difference in denaturing effect between urea and guanidine HCl described, the change in the titration curve in 4.6 M urea caused by the addition of guanidine HCl was measured as a function of the concentration of guanidine HCl. The addition of guanidine HCl was made to actin solutions containing 6 M urea to obtain a final urea concentration of 4.6 M and various concentrations of guanidine HCl. The results (Figure 13) show that the degree of normalization increases with an increase in the concentration of guanidine HCl.

A transitionlike titration curve similar to that obtained in 2 M guanidine HCl (Figure 10) could not be observed at any concentration of guanidine HCl in 4.6 M urea, presumably owing to a change in the structure by urea. The characteristic feature of the curves is the coincidence of titrations at 0.4 and 0.7 M guanidine HCl; that is, a certain portion of the buried tyrosines

were exposed below 0.4 M guanidine HCl, whereas the remainder became accessible to the solvent only when the concentration of guanidine HCl exceeded 0.7 M. These data were subjected to the same analysis as done before in Figure 11, namely, the fraction of tyrosines normalized at a constant *p*H was plotted against the concentration of guanidine HCl. Figure 14 shows the results at *p*H 9.9 and 10.6. In contrast to the result shown in Figure 11, no precipitation was observed upon the addition of a small amount of guanidine HCl in the presence of 4.6 M urea. At *p*H 10.6, approximately 65% of the tyrosines were normalized by 0.7 M guanidine HCl, followed by a sharp transitionlike

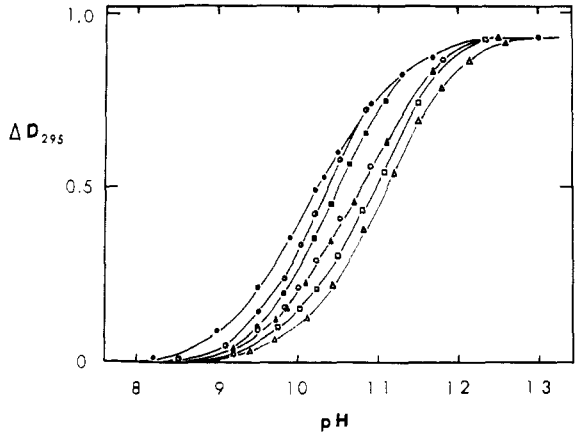

FIGURE 13: Spectrophotometric titration curve of actin in 4.6 M urea with various concentrations of guanidine HCl added. G-Actin was dissolved in 6 M urea and incubated at 0° overnight; then various amounts of guanidine HCl were added and the mixture was incubated at 0° overnight. Final concentration of urea was 4.6 M. Reference solution, 4.6 M urea (*p*H 7.5). Δ, 4.6 M urea; ●, 5 M guanidine HCl; ⊏, 4.6 M urea + 0.1 M guanidine HCl; ▲, 4.6 M urea + 0.4 M guanidine HCl; ■, 4.6 M urea + 1.0 guanidine HCl; ◑, 4.6 M urea + 2.0 M guanidine HCl. Protein concentration 0.25 mg/ml. The ordinate is the optical density at 295 mμ, ΔD_{295}, per gram of protein per liter.

complete normalization caused by further addition of guanidine HCl. This result suggests that there are two kinds of abnormal tyrosines, as pointed out from the results in Figures 10 and 11, and urea seems to normalize the weakly abnormal tyrosines. On the other hand, such a sharp increase in normalization could not be obtained at *p*H 9.9, and normalization was not complete at 2 M guanidine HCl. The disruption of the structure by urea at this *p*H (9.9) is not extensive enough to normalize all the buried tyrosines at 2 M guanidine HCl, resulting in the broader curve shown in Figure 14. Of course, normalization was completed by further addition of guanidine HCl beyond 2 M.

Discussion

As shown in Figure 1, there is no contribution to the spectrum from tryptophan residues in the molecule when the same solution of neutral *p*H is taken as a reference. If measurements be carried out on the difference spectrum between G- and F-actin at the same *p*H, spectrum peaks corresponding to both tryptophan and tyrosine are expected to appear at various *p*H values, shown in experiments by S. Higashi and F. Oosawa (to be published). However, such peaks emerge near 291 mμ or 284 mμ, different from the 295-mμ wavelength at which the present experiments were carried out. In addition, the spectral perturbation by guanidine HCl or urea is negligible at 295 mμ (Wetlaufer, 1962). Therefore contributions to ΔD_{295} from a spectrum shift owing to a conformational change and from perturbations by solvent are small compared with the increases in absorption at 295 mμ following tyrosine ionization.

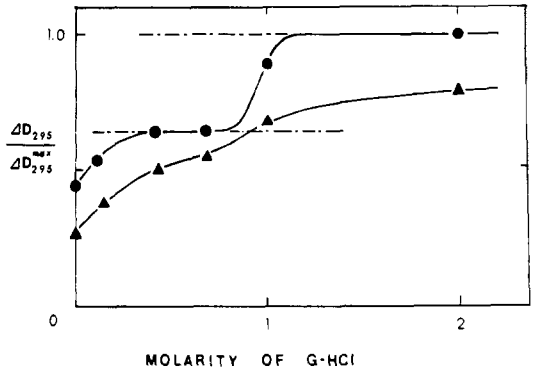

FIGURE 14: Plots of $\Delta D_{295}/\Delta D_{295}^{max}$ of the actin in 4.6 M urea and various concentrations of guanidine HCl versus the concentration of guanidine HCl at constant *p*H. ●, *p*H 10.6; ▲, *p*H 9.9. Reference solution is of the same concentration of protein and contains 4.6 M urea at neutral *p*H.

Although it is desirable to evaluate the intrinsic *pK* of the tyrosine ionization, there are two difficulties in its determination. First, the titration should be reversible in order to define a *pK* value (Tanford, 1962). As described previously, however, all titration curves in the present experiments were irreversible except in the presence of 5 M guanidine HCl. Second, the conformational change associated with depolymerization or with denaturation took place on the titration of F-actin or G-actin. Such changes in conformation and in the state of aggregation make it difficult to evaluate from the direct titration curve of actin a degree of ionization of the ionizable groups of the molecule (Tanford, 1962). Therefore only an apparent *pK* was estimated at the half-value of the maximum change at high *p*H. Furthermore, we cannot distinguish whether

a small increase observed at the initial stage of ionization is caused by a small degree of ionization of the buried tyrosines or by extensive ionization of a small number of normal tyrosines.

The *p*H dependence of the F to G transformation shows that it occurs around *p*H 10, as shown in Figure 3. Side-chain groups of amino acids which have their *pK* near this *p*H are the ϵ-amino group of lysine and the phenolic group of tyrosine (Edsall and Wyman, 1958; Tanford, 1962). It is possible that one or both of these amino acids are involved in the polymerization mechanism either directly or indirectly; i.e., once repulsion is enhanced at around this *p*H, depolymerization occurs following disruption of the interaction between molecules.

It is rather striking that, in spite of the absence of helix, abnormal tyrosines remain even in 6 M urea, as indicated by the forward and backward titration of Figure 12. One possible explanation is that helical parts in the molecule may be located near the surface, whereas deeply buried tyrosines interact with other side-chain groups (Kendrew, 1962; Hermans and Scheraga, 1961; Tanford, 1962) to maintain a rigid structure which presumably forms a core. Urea breaks the helical structure of the molecule, but is not strong enough to disrupt the interaction of tyrosines with other groups in the core, and the addition of guanidine HCl gives rise to unfolding not only of the helical region but also of the region where the stronger abnormal tyrosines are buried, a region which perhaps consists of hydrophobic amino acid residues (Kendrew, 1962). The classification of the abnormal tyrosines into two kinds is consistent with this explanation. Furthermore, the region where the abnormal tyrosines are contained is distinguishable from a region critical for the polymerization, since the loss of polymerizability occurs before normalization of the tyrosines.

At 30 mM KOH, there are two values for the molecular weight, according to treatment with or without guanidine HCl, corresponding to subunit and monomer. Since other physicochemical measurements did not show any difference between them, e.g., there is no helix and all the tyrosines are ionized in both, it is likely that the binding between subunits, which is presumably of hydrophobic character, is disrupted by guanidine HCl. In contrast, splitting of the monomer into subunits does not occur in urea solutions. Therefore this is a good example showing the difference between the denaturing actions of guanidine HCl and urea, both having been suggested as hydrophobic bond breaking reagents (Kauzmann, 1959; Donovan *et al.*, 1959).

As described, guanidine HCl seems to have a special effect on side-chain interactions in an actin molecule. Another effect, i.e., polymerization caused by the addition of a small amount of guanidine HCl (urea has no such effect), seems to be of interest. A study along this line is now in progress.

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