Remasking of Hidden Tyrosines of Human Serum Albumins after Exposure to High and Low pH†

Jacinto Steinhardt* and Nancy Stocker

ABSTRACT: The refolding of human serum albumin after unfolding (denaturation) by both acid and base may be followed by a stop-flow method which depends on distinguishing immediate from time-dependent changes in the ionization of its constituent tyrosines at pH values over 10.8 (Steinhardt, J., and Stocker, N. (1973), *Biochemistry 12*, 1789). Maximal unfolding at 25° and 0.10 ionic strength occurs at pH <3.3. The abnormally high p*K* (*ca.* 11.3) of the accessible tyrosines of the native conformation is normalized by the N → F isomerization which occurs at pH <4.5; the abnormal p*K* is not immediately restored when the pH is raised. The unfolded states produced by high and low extremes of pH are shown to differ. Greater extremes of pH are required to unfold at 0.03 ionic strength than at 0.1 or 0.24; 20–50 equivalents of sodium dodecyl sulfate, which are known to reduce the extents of unfolding at both high and low pH, have little if any effect on the *rates* of refolding; the large effects they have on the *unfolding* rates are confirmed. The rates of refolding after denaturation by acid have little dependence on the pH of refolding at pH >10.8, but the refolding rates after alkaline denaturation depend strongly on pH. The method should be applicable to other means of unfolding and to other proteins.

We have reported the results of stop-flow experiments in which the pH of solutions of human serum albumin is suddenly raised, with or without the simultaneous addition of sodium dodecyl sulfate. These experiments have permitted determination of the number of tyrosine side chains exposed to solvent in the native state, and the number exposed at equilibrium at higher pH, in the presence of various amounts of detergent (Steinhardt and Stocker, 1973). The kinetics of the unfolding process has also been reported, as a function of pH and detergent concentration. The method depends on the fact that the optical density at 243 nm *at zero time* when the pH is raised is a measure of the number of tyrosines already exposed in the native protein at pH 5.6; the optical density at any time thereafter, including final equilibrium, shows the number exposed at the ambient pH, which at pH $\geq$10.8 includes effects of unfolding.

By adding the detergent *before* raising the pH to 11.4, unfolding caused by the detergent was observed even in the vicinity of the isoionic point, although this unfolding could not affect the ionization of the tyrosines at this pH. It was also shown that when the initial pH was $\ll$5.6, the zero-time optical density at pH 11.7 was increased but the optical densities at infinite time were essentially the same as when the initial pH was 5.6.

It thus became clear that the unfolding produced in the acid isomerization reaction (N → F transition) could be studied by bringing human serum albumin solutions to various acid pH conditions and then suddenly raising the pH to a convenient alkaline value at which extrapolations of optical density at 243 nm to *zero time* could be made accurately and conveniently. This paper reports the results of such a study.

Experimental Section

Details of the materials and experimental procedures have already been given (Steinhardt and Stocker, 1973). Protein solutions were acidified with HCl at 0.10 ionic strength (NaCl) in the presence of various quantities of sodium dodecyl sulfate. The solutions (usually 0.4% protein) were then transferred to the Gibson-Durrum stop-flow apparatus and mixed with equal volumes of buffers that would bring all the solutions to the same pH at 0.24 ionic strength. When it was desired to study the regeneration of alkali-denatured human serum albumin, the protein was initially exposed to an alkaline buffer (tertiary phosphate) or dilute NaOH at 0.24 ionic strength for measured intervals of time, and then brought in the stop-flow apparatus to a lower pH (high enough for observation of changes in tyrosine ionization) by addition of HCl plus salt. The time-dependent diminution of absorption was recorded, including the value at final equilibrium.

All of the experiments were performed at 25.0 ± 0.2°. The temperature in each experiment was constant to better than 0.02°.

Results

Regeneration of Human Serum Albumin Unfolded at High pH. It has been shown (Steinhardt and Stocker, 1973) that 0.1% human albumin brought to pH 12.09 at 0.24 ionic strength has an optical density at 243 nm of 0.61 when viewed in a 2-mm cuvet.[1] This absorbance is due to the complete ionization of all 18 tyrosines in the molecule plus a constant contribution (independent of pH) of 0.14 from the other chromophores of the molecule. When unfolded at pH 12.02 for 4–5 min and returned "instantaneously" in the Gibson-Durrum stop-flow apparatus to pH 10.87, the *initial* (zero elapsed time) absorbance is 0.414 rather than 0.61 because only 58 percent of the tyrosines are ionized at pH 10.89. In less than a second the absorbance decreases to 0.267, *i.e.*, there is a further loss of absorbance at this pH of 0.147 which corresponds to the masking of about 10 out of 18 tyrosines;

† From the Department of Chemistry, Georgetown University, Washington, D. C. 20007. *Received February 26, 1973.* Supported by National Science Foundation Grant No. GB 13391.

[1] The values cited in the paper are as given by the stop-flow apparatus and are all corrected to 0.1% HSA. They must be multiplied by 1.18 to represent actual optical densities as determined in a Cary spectrophotometer.

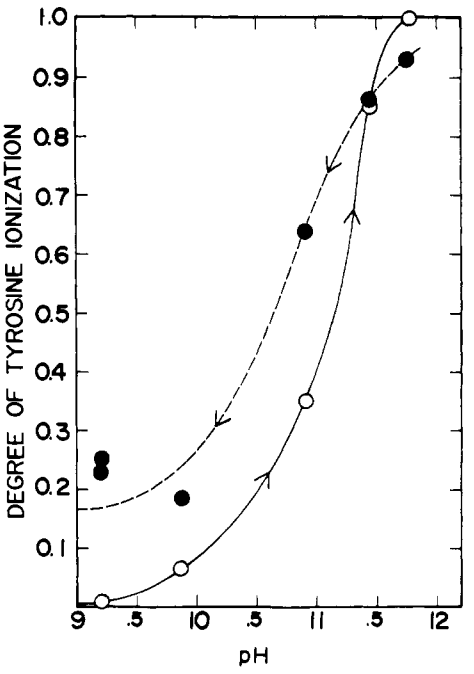

FIGURE 1: The instantaneous apparent degrees of tyrosine ionization of solutions of human serum albumin brought to the pH values indicated before time-dependent changes ensue: upper curve, initial pH 12.02; lower curve, initial pH 5.6. All data are at 25° and 0.24 ionic strength.

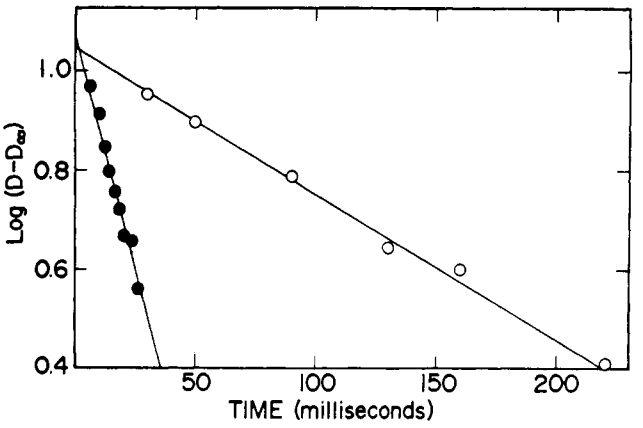

FIGURE 2: Kinetics of refolding at pH 9.22 (lower curve) and 9.86 (upper curve) after unfolding at pH 12.02 at 25° and 0.24 ionic strength. The ordinate (logarithm of density minus final density) is in arbitrary units.

this number is close to that in our earlier paper (12) which was obtained from the ratio of accessible to total tyrosines in the native molecule at pH values below about 10. The unmasking brought about by the brief exposure to pH 12.02 is thus largely reversible.

We may therefore proceed to obtain a back-titration curve of all the tyrosines in human albumin by first bringing the human albumin solution to pH 12.02 and then immediately mixing this alkaline solution in a series of stop-flow experiments which bring the solutions to a number of lower pH values. The results of such experiments are shown in Figure 1 together with data obtained by raising the pH from an initial value of 5.6. Both are presented in terms of equivalent degrees of ionization *of all 18 tyrosines*. Both sets of data represent zero-time values and are therefore due to ionization of initially exposed groups only, exclusive of changes caused by refolding or unfolding which later ensue. The two curves differ because the titration toward alkaline pH shows only the partial ionization of the initially accessible tyrosines at each pH, while the back-titration (initial pH 12.02) represents the ionization of *all* the tyrosines at every pH, as in a pure titration curve, uncomplicated by unfolding equilibria. Inspection of the back-titration curve indicates that its midpoint (50% ionization) is about 10.6, about 0.4 pH unit lower than the corresponding midpoint value which results from the forward titration starting from pH 5.6. The back-titration curve is similar to the normal result for a set of groups having a single intrinsic pK, and no important electrostatic interactions. The forward titration becomes steeper (more "cooperative") as the pH rises, because titration of accessible groups is mixed with an increasing number of titratable groups that also depend on pH (Steinhardt and Reynolds, 1969).

When the exposure time at high pH (12.02) is increased from 4–5 to 25 min, a slightly higher absorbance, 0.45, is found at zero time at pH 10.8 but irreversible changes appear to have occurred because the lowest value to which the absorbance returns at pH 10.8 is 0.32 rather than 0.27; the difference corresponds to the *irreversible* unmasking of at least three tyrosines at this pH. With still longer periods at pH 12.02 larger proportions of the tyrosines remain permanently accessible and partially ionized at pH 10.89.

The presence of 20 equiv of sodium dodecyl sulfate does not greatly change these results, although the velocity of the refolding reaction is affected (see next paragraph). With 40 equiv there is a somewhat greater degree of refolding; the precise amount is not easily determined because of the effect of the bound detergent anions on the ionization of the protein tyrosine. The larger extent of refolding noted is to be expected, in view of the protective effect of these quantities of dodecyl sulfate noted in our previous paper from both our earlier work (Steinhardt and Stocker, 1973) and that of other laboratories (*e.g.*, Aoki, 1958; Lovrien, 1963; Markus *et al.*, 1964).

Examples of the kinetics of the refolding reaction are shown in Figure 2. The reaction isotherm is clearly first order over a large part of its course (Steinhardt and Stocker, 1973). The reaction velocity depends on the pH and is very fast (half-period = 17 msec) at pH 9.22 where no unfolding was detectable. It is much slower, however, than the unfolding reaction when carried out at higher pH (100 msec at pH 9.86 and much longer at pH 10.8 and above, where substantial unfolding was seen). The great diminution in rate at higher pH values is at least in part the result of the decrease in the supply of protons which must be captured by ionized tyrosine for refolding to occur. The effect of the presence of dodecyl sulfate on the refolding rate has not yet been determined.

Unfolding by Acid (N → F Isomerization). The extent to which the tyrosines of human albumin are exposed at pH values below the isoionic point may be determined by an extension of the stop-flow technique. Protein is brought to the desired acid pH values for controlled intervals of time and then suddenly brought to the same constant viewing pH, 10.8, as was used in the observations of refolding after unfolding by base. The choice of viewing pH is quite critical since there is no ionization of tyrosines *in the native state* at pH values as high as 9.8, and since small amounts of unfolding occur even at pH values as low as 10.8. Thus when very little unfolding is brought about by acid, as for example, at pH 4.8, the absorbance found at zero time at pH 10.8 may be actually less, rather than more, than the absorbance found at equilibrium, *i.e.*, *additional* unfolding occurs at the viewing pH.

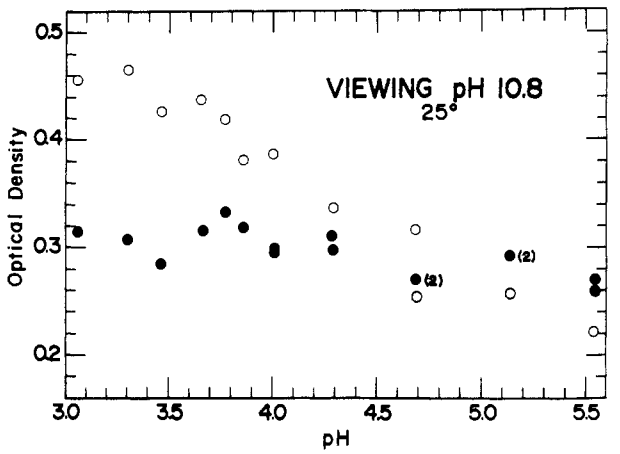

FIGURE 3: The extents of unfolding at the pH values indicated measured by reading the optical densities at pH 10.87 at 25° and 0.24 ionic strength. Open circles represent instantaneous values; closed circles represent equilibrium (infinite time) values. The ionic strength at the pH values indicated was 0.10.

Figure 3 illustrates the results at pH 10.8 after acid unfolding. The zero-time values (upper curve) show that more and more unfolding occurs as the pH is lowered, at least as far as pH 3.1. There are no obvious discontinuities at any intermediate pH. On the other hand, the equilibrium absorbances (lower curve) differ very little, regardless of the acid pH, and represent almost complete reversal of the unfolding. The figure shows that small amounts of unfolding occurred at the viewing pH when the pH previously used was relatively high (4.0–4.6).

The highest zero-time absorbance found in a 2-mm path cuvet was 0.47, rather than the 0.61 found when the protein was unfolded at pH 11.67–12.05. The difference is due to the incomplete ionization of tyrosine at pH 10.8. Even if all the tyrosines become accessible at the most acid pH values, their ionization would be incomplete at pH 10.8. Thus, for example, it has been shown (Figure 1) that after unfolding at pH above 12, the instantaneous optical density on changing the pH to 10.8 is only 0.414. Regardless of whether the alkaline and acid unfolding are both completely reversible the latter figure should then be the highest initial absorbance at pH 10.8 obtained after exposure to any pH in Figure 3. The highest value found, 0.47, is significantly above this figure. The discrepancy is made use of in the Discussion to draw important conclusions as to the nature of the N → F isomerization.

When the exposure to acid is moderate at 4.02 or 4.55 no refolding can be seen at pH 11.38; instead, an additional small amount of unfolding is produced before equilibrium is reached. It is clear that the lower pH, 10.8, is more suitable for the determination of unfolding by acid, and especially for the observation of refolding. Even at a pH as low as 10.87, however, *in the absence of detergent*, refolding is not complete, as can be seen by slight amounts of time dependent refolding at the viewing pH when only small amounts of acid unfolding occurred.

A direct comparison of the amounts of tyrosine exposure brought about in the acid isomerization and in the unfolding by high pH can best be made by comparing the zero time optical densities of solutions exposed to pH 3.01–3.30 and then brought to pH 10.8 (Figure 3) with zero time optical densities of solutions unfolded at pH 12.1 and then brought to pH 10.8 (Figure 1 after translation into optical density units, ODU). The ODU averages (four experiments similar to Figure 3) are 0.42 after exposure to pH 3.01–3.30 and 0.44 after 12.1. Thus the amount of unfolding at acid pH is about as great as at 12.1. Since all the tyrosines appear to be accessible after exposure to 12.1 (Steinhardt and Stocker, 1973), they must be equally accessible at acid pH (pH 3–3.3) if their p*K* after unfolding is "normal" in both these cases.

TABLE I[a]

Viewing pH	Initial pH: pH 5.6	Initial pH: pH 3.75
10.97	0.17 (0.22)	
11.06		0.22 (0.20)
11.43	0.27 (0.33)	
11.51		0.33 (0.33)
11.67	0.36 (0.40)	
11.65		0.33 (0.36)

[a] The figures in parentheses represent the absorbances at equilibrium. The differences between the figures in parentheses and the numbers next to which they are placed in the pH 5.6 column represent unfolding; and either refolding or further unfolding in the pH 3.75 column.

The unfolding in acid appears to be slightly less fully reversible than the unfolding at 12.1. This is shown by the recovery of an average of 0.121 ODU at 10.8 after acid unfolding (pH 3.0–3.3) as compared with a recovery of an average 0.147 ODU after unfolding at pH 12.1. The slightly higher optical densities at pH 10.8 after unfolding at pH 12.1 support this inference.

There is no sign in Figure 3 of a separation, with respect to pH, of the acid unfolding process into two steps, *i.e.*, isomerization at pH >4 and "swelling" (which alters the viscosity) at pH <4. The exposure of tyrosines to solvent is gradual over the entire range down to at least pH 3.3.

Effect of Ionic Strength on Acid Unfolding. All of the exposures to acid in the experiments described above were made at an ionic strength of 0.10 (NaCl). The ionic strength at the viewing pH was 0.24. When the acid exposures are made at a very much lower ionic strength, 0.002, and the viewing pH is also at a low ionic strength, 0.032, considerably lower zero time absorbances are found even when higher viewing pH values are resorted to. It might appear therefore that the N → F transition does not expose all the hidden tyrosines when the ionic strength is as low as 0.032. However account must be taken of a lower degree of ionization of the accessible tyrosines at the viewing pH at low ionic strength: zero-time optical densities on suddenly raising the pH of solutions at this ionic strength from 5.6 to 10.8 are approximately 0.17, only 0.03 higher than the 0.14 non-tyrosine absorbance which is independent of pH. Comparisons are best made, therefore, at higher viewing pH values.

Table I shows the zero-time optical densities observed when solutions of human albumin are brought to various pH values of 0.032 ionic strength, from pH 5.6, or from pH 3.75 where acid unfolding occurs.

At this ionic strength ionization of *exposed* tyrosines appears to be complete at about pH 11.5 (at 0.24 ionic strength it appeared to be almost complete at 11.67). If this conclusion is correct, little acid unfolding appears to have occurred at low ionic strength, *i.e.*, the zero-time values in the pH 3.75 column are very close to those in the pH 5.6 column.

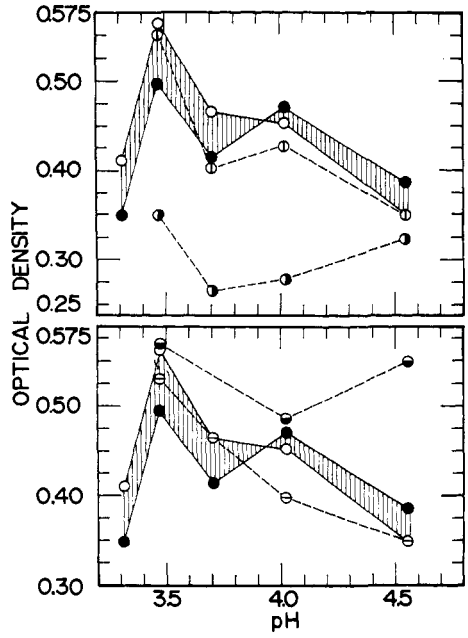

FIGURE 4: The effect of two different levels of sodium dodecyl sulfate on the extent of unfolding by acid, when monitored at pH 11.36. The open and filled circles joined by cross-hatching represent, in both panels, the zero time and infinite time results in the absence of detergent. Top panel: (◑) instantaneous values of optical density in presence of 20 equiv of detergent; (◑) equilibrium values (long time) with 20 equiv (a single point at pH 3.70 was obtained with 30 equiv). Bottom panel: (⊖) instantaneous value in presence of 200 equiv of dodecyl sulfate; (◒) equilibrium value.

Effects of Sodium Dodecyl Sulfate on Acid Unfolding. It has been shown that sodium dodecyl sulfate can either stabilize proteins against denaturation at high pH, or induce or enhance denaturation when the amounts bound by the protein exceed 12–20 equiv (Steinhardt and Stocker, 1973). In order to determine whether there are similar effects when human albumin is unfolded by acid, the same kind of data displayed in Figure 3 has been obtained with various amounts of dodecyl sulfate present at a number of different initial unfolding pH values.

Figure 4 shows the results. Although measurements similar to those just described have been made in the presence of 10, 20, 30, 50, 100, 200, and 300 equiv of sodium dodecyl sulfate, as well as in its absence, the effect of the detergent on the acid unfolding can be practically completely characterized by describing the results with a "protecting" concentration, 20 equiv (top panel of figure), and with an "unfolding" concentration, 200 equiv (bottom panel), together with the data obtained in the total absence of this substance. All of the data in Figure 4 have been obtained at a relatively high viewing pH, 11.36 ± 0.02, for reasons which will become clear below.

As the top panel of Figure 4 shows, the higher viewing pH has resulted, in the absence of detergent (boundaries of cross-hatched area), in higher zero-time absorbances after exposure to all acid pH values than were found at the lower viewing pH, 10.8 (Figure 3). Also, much less recovery or refolding at infinite time is realized; in fact when the initial acid unfolding was not maximal, raising the pH to 11.36 caused no recovery, but instead produced a small increase in the degree of unfolding already produced by acid (note the crossing of the boundary lines of the cross-hatched area near pH 3.9). The addition of 20 equiv of dodecyl sulfate has little if any significant effect on the amount of unfolding produced by acid. It has a profound effect, however, on the amount of refolding that ensues

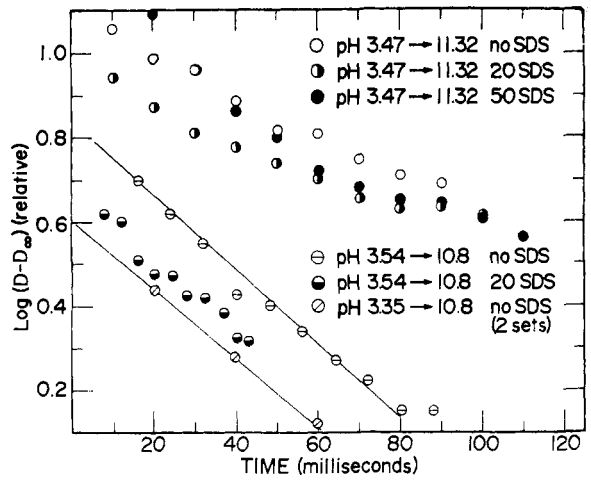

FIGURE 5: Selected examples of kinetics of refolding after unfolding by acid: open circles, no dodecyl sulfate; closed circles, 20–50 equiv dodecyl sulfate. Lower portion, pH raised to 10.81 from 3.35 and 3.54; upper portion, pH raised to 11.32 from 3.47.

when the pH is raised to 11.36. The effect is practically all accounted for by the reduction in rate of unfolding brought about by dodecyl sulfate which Steinhardt and Stocker (1973) have already reported.

This conclusion is reinforced by the data in the bottom panel of Figure 4 which shows the effects of the presence of 200 equiv of dodecyl sulfate. The cross-hatching again shows the results in the absence of detergent. It will be seen that the instantaneous values of the absorbance are only slightly changed from their values in the presence of 20 equiv, nor are they radically different from the values in the absence of detergent. The final values, however, are greatly elevated: unfolding has continued after the pH was raised; the optical density is higher than in the absence of detergent at every pH, and never lower than about 0.49. Again, the simplest interpretation is that low concentrations of dodecyl sulfate have only a minor effect on the N → F transition, but a powerful influence on unfolding at higher pH.[2]

Kinetics of Refolding after Unfolding by Acid. An analysis of the complex time course of refolding is least ambiguous when limited to those reactions in which the largest amounts of refolding occur, *i.e.*, decreases of at least 0.07 ODU when dodecyl sulfate is absent, and of 0.136–0.200 ODU, in the presence of 20–40 equiv of detergent. An examination of *refolding* cannot be made when the dodecyl sulfate concentration or the pH is so high that additional time-dependent increases in absorbance occur on raising the pH *after* an initial exposure to pH <4.0. Thus, rates of refolding at pH 11.36 as affected by concentration of detergent can be examined meaningfully only where refolding rather than further unfolding occurs. One is limited, therefore, to data obtained with 20–50 equiv of detergent, or, in the absence of detergent, to pH values at or below about pH 4.0.

Selected examples of the early stages of the complex course of the refolding reaction are given in Figure 5. In this figure the ordinates are not directly comparable since they depend on the amount of refolding which occurs; thus the values of the ordinate have been adjusted to avoid overlapping of the several sets of data. A first-order process seems to prevail for a

[2] Aoki (1958) found that a cationic detergent, dodecylpyridinium bromide, affected the N → F transition in acid solutions although sodium dodecyl sulfate did not.

half (pH 11.3) or more (pH 10.8) of the course of the reaction. Unlike refolding after alkaline unfolding, the velocity of this first-order process does not appear to have a strong dependence on the unfolding pH (thus the extent of unfolding). This may also be true of the slower processes which follow it. There is also only a slight dependence of the initial velocity on the viewing pH (10.8 or 11.3).

The effect on the velocity of 20 equiv of dodecyl sulfate, which enhances the *extent* of refolding, is also small in the same range shown in Figure 5. Thus its effect on the equilibrium must depend largely on the retardation of the unfolding reaction by small amounts of dodecyl sulfate.

At higher pH values, or at higher concentrations of detergent, the latter accelerates the change in optical density to a much greater extent than is shown in Figure 5, but under these conditions the sign is reversed, *i.e.*, further unfolding, rather than refolding, occurs.

Discussion

Experiments similar to the kinetic aspects of the present investigation have been reported for ribonuclease A and chymotrypsinogen by Tsong *et al.* (1971) and Tsong and Baldwin (1972), who have analyzed their kinetic data in terms of a sequence of progressively slower first-order reactions, involving a succession of intermediate states. Such states have been directly demonstrated with horse ferrihemoglobin by Polet and Steinhardt (1969) by running parallel kinetic experiments at a large number of wavelengths. Such analyses have not yet been applied here.

The results presented in this paper serve rather to demonstrate that the instantaneous ionization of the tyrosines at high pH, as well as the time-dependent change in this ionization which follows, may be used to illuminate changes in protein conformation even when they occur at acid pH. Some of the information gleaned by application of this method is briefly enumerated below. It has been shown that higher instantaneous absorbances are found at the viewing pH values, 10.8 and 11.36, after exposure to acid pH than can be explained by the expected degree of ionization of *all* the tyrosines in human albumin at those pH values if the intrinsic pK values used are those of the native protein, *i.e.*, 11 or higher, as deduced in our earlier paper (Steinhardt and Stocker, 1973), and also reported by Avruch *et al.* (1969). The data of Avruch and his collaborators also showed that this anomalously high value for tyrosine ionization in a protein was reduced by more than one pH unit, becoming normal, when the amino groups of the lysines of bovine serum albumin were acetimidated. Steinhardt and Stocker (1973) observed that these changes and certain other aspects of the titration curve of bovine albumin with base were consistent with strong interactions between the amino groups of the lysines and most of the tyrosines in the native protein, interactions which were apparently greatly weakened by acetimidation. If the N → F transition also destroys or weakens this interaction,[3] even though it may do so reversibly, then the zero time readings of optical density at the viewing pH will be higher than those predicted by use of the apparent pK of tyrosine obtained by spectrophotometric titration of the native protein with base. The fact that higher values of absorbance are found at the viewing pH than can be accounted for by the tyrosine pK of the native protein suggests strongly that the N → F isomerization involves abolition of the strong tyrosine–lysine interaction of the N form. This conclusion is compatible with Foster's view of the mechanism of the N → F process (Foster, 1960) as one which separates numerous paired charges (presumably lysines and the decarboxylic amino acids) from one another.

It appears also that refolding at pH $\geq$10.8 after acid unfolding has a characteristic initial rate at 25° which depends very slightly, if at all, on the unfolding or refolding pH values (over the range investigated) or on the presence of 20–50 equiv of dodecyl sulfate. Both pH and dodecyl sulfate concentration, however, have profound effects on the final equilibrium at either low or high pH. Their effects on the latter therefore must be largely due to their effects on the unfolding reaction which we have shown to be large (Steinhardt and Stocker, 1973). The folding rate process appears to be dominated by a built-in program, the amino acid sequence. The very minor effect of pH may be due to the remoteness of the refolding pH region from the pH range in which acid unfolding is brought about. At a still higher pH the rate may fall more noticeably since the supply of protons, on which it partly depends, will fall by a larger factor.

The initial rates of refolding after unfolding at high pH (>12) depend very strongly on pH, and therefore cannot be directly compared with refolding rates after acid denaturation. They are very fast when refolding takes place at pH values below about 9.8; but in the pH range in which refolding after acid treatment has been studied, the rates of recovery from high pH are much slower. Clearly the two unfolded states (acid and alkaline) differ, and the path to refolding must therefore differ also.

Since the unfolding of proteins can be brought about with various agents (high temperature, guanidine, salt, urea, environmental changes, among others) there may be numerous other opportunities to apply the technique used in this paper. Application to another protein should have a high priority, in order to establish its generality.

[3] As, for example, by protonating lysines which may be masked in the N form, as many of the carbonyls are (Vijai and Foster, 1967).

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