Application of the pH-Jump Method to the Titration of Tyrosine Residues in Bovine α-Lactalbumin†

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ABSTRACT: A stopped-flow technique has been developed for the *zero-time* spectrophotometric titration of tyrosine residues in the purely native or in the purely alkaline denatured state of α-lactalbumin that undergoes an alkaline conformational transition in the pH region of tyrosine ionization. The progressive absorption change at 298 nm caused by a pH jump from neutral pH is shown to result from the change in ionization of the tyrosine residues brought about by a first-order process of the conformational transition. Extrapolation to zero time gives the titration curve for purely native α-lactalbumin. Similarly, the pH jump from highly alkaline pH gives the titration curve for the purely alkaline denatured protein. The method should be generally applicable to other proteins that contain tyrosines. Analysis of the titration curves suggests that the four tyrosines in native α-lactalbumin have pK values of 10.5, 11.8, 11.8, and 12.7, respectively. After the alkaline transconformation, all of them become titrated normally with a pK value of 10.3. A comparison of these results with the ionization behavior of tyrosines in hen egg white and human lysozymes is presented and discussed in terms of differences in the sequences of the proteins.

Anomalous tyrosine ionization behavior has been observed in many proteins in the native state. The characteristics of such anomalous ionization are generally regarded as reflections of the secondary and tertiary structures of the proteins. The investigation of tyrosine residues in proteins then has been widely used as a probe of protein structures; the principal approaches include spectrophotometric and electrometric titration, solvent perturbation spectroscopy and chemical modification (Kronman & Robbins, 1970). Spectral changes occurring on tyrosine ionization exhibit a typical difference absorption peak having a maximum close to 295 nm, and the spectrophotometric titration curve may provide a clue as to the accessibility of tyrosine residues in proteins (Donovan, 1973). Nevertheless, in many proteins, conformational changes, which are too rapid to follow by usual spectrophotometric techniques, also occur in the pH region of tyrosine ionization, and these preclude any determination of the ionization behavior of tyrosines in the native state at least by the equilibrium methods. In such cases, stopped-flow pH-jump techniques should be applicable and provide an effective approach to the above problem, whereas such an approach has not been widely used.

Steinhardt & Stocker (1973a,b) have first applied the stopped-flow techniques for distinguishing between tyrosines of human serum albumin which are freely accessible to solvent and those which are hidden in the interior of the native molecule. Garel & Baldwin (1975) have studied the refolding of nitrotyrosyl ribonuclease A and detected a pK change of nitrotyrosyl groups on refolding by the pH-jump method. Hiromi et al. (1975) have also studied kinetically the alkaline conformational changes of α-amylase by monitoring changes in ionization of tyrosines. However, all of these research groups were not primarily concerned with the titration of the tyrosine residues, but rather with the unfolding and refolding processes of the proteins. Kurz & Holtzer (1977) have reported the titration of tyrosines in undissociated myosin by utilizing a kinetic method, but they neglected the effects of rapid conformational changes on tyrosine ionization.

The present study shows that the application of the stopped-flow pH-jump method is profitable for the quantitative analysis on the titration of tyrosine residues in the proteins that undergo fast alkaline transconformations. The basic concepts in this approach are as follows. When the native protein is exposed to an alkaline solution by a pH jump, the absorption changes are caused by the three different events, namely: (i) the instantaneous change in tyrosine ionization of the native molecule after alkalization, but before the onset of conformational transitions (we denote such absorption change as δA_{ion}); (ii) the spectral blue shifts for aromatic residues caused by alkaline transconformations themselves (δA_{conf}); and (iii) the alkaline transconformations also cause the concomitant changes in the intrinsic tendency of tyrosine ionization, and such changes are also observed as the absorption changes ($\delta A_{conf\text{-}ion}$) (Figure 1). Since the first event (δA_{ion}) is completed within the dead time of the stopped-flow apparatus (Eigen, 1964), we can only observe δA_{conf} and $\delta A_{conf\text{-}ion}$ as the time-dependent absorption changes. Then, if there is no conformational change that is too rapid to measure by the stopped-flow experiments, extrapolation of the observed absorption changes to zero time gives information about ionization equilibrium in the native state at the alkaline pH, and the spectrophotometric titration curve of the native molecule can be extended over the pH regions of the alkaline transitions; in the following, the titration curve obtained by these methods is termed the zero-time titration curve. Similarily, the absorption changes caused by the pH jump from highly alkaline pH can also be classified into the above three categories, and the same extrapolation gives the zero-time titration curve for the purely alkaline denatured protein.

The application of the above methods was done on bovine α-lactalbumin, which has the primary chemical structure very similar to that of lysozyme (Brew et al., 1970). Many of the comparative studies have emphasized the similarity in the tertiary structure between the two proteins, but several differences in their properties have also been described (Sugai et al., 1973; Hill et al., 1974; Kuwajima et al., 1976). α-Lactalbumin is known to undergo a fast alkaline denaturation in the pH region of tyrosine ionization (Robbins et al., 1967; Kronman et al., 1967). This is in contrast to the facts that lysozyme is unusually stable against pH and that its denaturation occurring at pH >12 is very slow (a relaxation time of a few hours; Inada, 1961; Imoto et al., 1972). Because of these facts, the ionization behavior of tyrosines in native lysozyme has now been well characterized by usual spectrophotometric titration techniques without the utilization of

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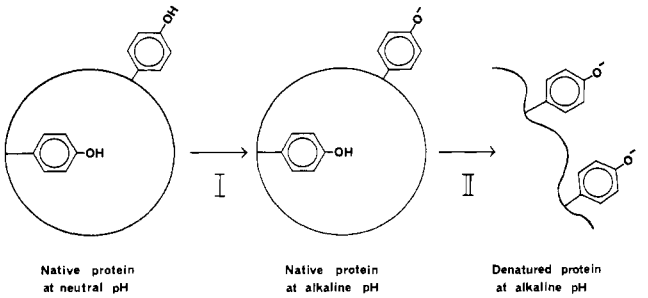

FIGURE 1: Schematic representation of alkalization of a protein from the native state. The process I corresponds to the instantaneous ionization of the tyrosine originally exposed to solvent in the native state and involves the absorption change of δA_{ion}. The process II is the transconformation process, the tyrosine residue buried in the native molecule becomes exposed to solvent, and this process involves two kinds of absorption changes, δA_{conf} and $\delta A_{conf\text{-}ion}$.

the fast kinetic methods (Tojo et al., 1966; Latovitzki et al., 1971). For native α-lactalbumin, on the other hand, the occurrence of the fast alkaline denaturation makes it difficult to determine the ionization behavior of tyrosines by such usual spectrophotometric techniques.

Materials and Methods

Materials. Bovine α-lactalbumin, Gdn·HCl,[1] and other chemicals used here were the same as in the previous studies, and the concentrations of the protein and Gdn·HCl were also determined as reported previously (Kuwajima et al., 1976).

Methods. The kinetic measurements of the pH jump were carried out in a stopped-flow rapid reaction analyzer (Union, RA-1100) with a cell of 10-mm path (Kita et al., 1976; Nitta et al., 1977). The dead time of the apparatus (usually 2 ~ 3 ms) was determined precisely in each experiment by use of 2,6-dichlorophenolindophenol and L-ascorbic acid (Tonomura et al., 1978). Difference spectra measurements were done on a Hitachi Perkin-Elmer 124 spectrophotometer. CD spectra were measured in a Jasco J-20 or in a Union CD-1000 spectropolarimeter. Measurements of pH were made with a Hitachi-Horiba Model 7 pH meter, which was standardized by the buffers and by 0.1 N NaOH.

In most of the experiments, protein solutions contain 0.5 M Gdn·HCl, 0.1 M KCl, and suitable buffers. At 0.5 M Gdn·HCl, the protein is known to be completely native in the neutral pH region (pH >5) (Kuwajima et al., 1976). Since long time holding of the protein solution at highly alkaline pH causes an irreversible transition, all the measurements had been done within 15 min after preparation of the protein solution.

All the kinetic data were fitted by the method of least squares to a first-order rate law

$$A_t - A_0 = (A_\infty - A_0)(1 - e^{-kt}) \qquad (1)$$

where A_t is the absorption value at time t after the pH jump; A_∞ and A_0 are the values at infinite and at zero time, respectively; and k is the first-order rate constant. For evaluation of the zero-time titration curve, the quantity $A_\infty - A_0$ rather than k is essential, and we use the term "kinetic amplitude" for $A_\infty - A_0$ in the following.

Results

Equilibrium of the Alkaline Denaturation of α-Lactalbumin. The alkaline transconformation of α-lactalbumin can be observed by near-ultraviolet CD spectra (Robbins & Holmes, 1970). Ellipticity bands above 260 nm are remarkably reduced with increasing pH above 10. Figure 2 shows the ellipticity at 270 nm as a function of pH in the presence of 0.5 M Gdn·HCl. The protein is in the native state at pH 9.2 and in the denatured state at pH 11.5.

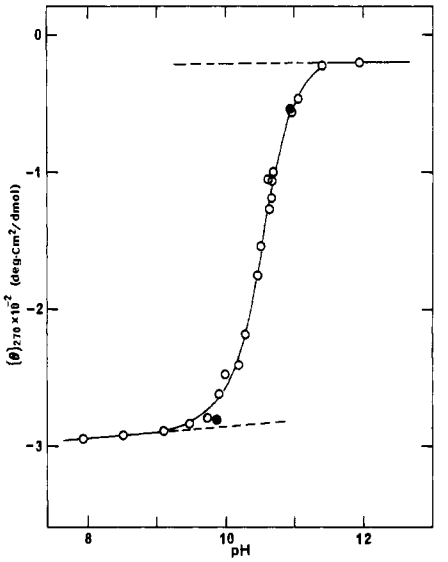

FIGURE 2: The dependence of the ellipticity at 270 nm on pH at 0.5 M Gdn·HCl and 25.0 °C. Protein concentration is ca. 0.05%, and path length of the optical cell is 10 mm. Filled circles represent reversals from alkaline pH.

The alkaline ultraviolet difference absorption spectrum relative to neutral pH was found to be expressed as a superposition of the tyrosyl ionization spectrum and a blue-shifted spectrum of aromatic residues caused by the denaturation, as previously shown by Kronman et al. (1967).

Difference Absorption Spectrum Caused by the Alkaline Denaturation Itself. For the characterization of tyrosine ionization in the protein from the absorption spectrum, it is convenient to separate the spectrum into the ionization spectrum and the spectrum caused by the alkaline denaturation itself. In order to obtain the latter spectrum, the pH-jump experiments were carried out from the highly alkaline to the neutral pH. At the initial pH (11.6) the protein is in the purely alkaline denatured state. At the final pH (8.0), the protein is in the native state (Figure 2) and in addition all the tyrosine residues are completely protonated irrespective of the conformational states (cf. ionization curves of tyrosines in Figure 4), which results in $\delta A_{conf\text{-}ion} = 0$. Then we can observe only δA_{conf} as the time-dependent absorption change. A single first-order rate process was found to dominate the kinetics, and the relaxation time of the process (~4 s) is independent of observation wavelength, while the kinetic amplitude was found to remarkably depend on wavelength. The wavelength dependence of the observed kinetic amplitude gives the difference spectrum for the alkaline transconformation without any contribution of tyrosine ionization. The resultant difference spectrum shown in Figure 3 exhibits the characteristics for the denaturation of α-lactalbumin, and at 298 nm the difference absorption in the spectrum is essentially zero. Thus, the absorption change at 298 nm can be taken as a measure of tyrosine ionization.

Tyrosine Ionization under the Denaturation Equilibrium. The pH dependence of the equilibrium difference extinction coefficient at 298 nm, $\Delta\epsilon^{eq}_{298}$, is shown in Figure 4 and gives the ionization curve under the equilibrium of the alkaline denaturation of the protein. As seen from Figure 2, however, both the native and the alkaline species of the protein par-

[1] Abbreviations used: Gdn·HCl, guanidine hydrochloride; CD, circular dichroism.

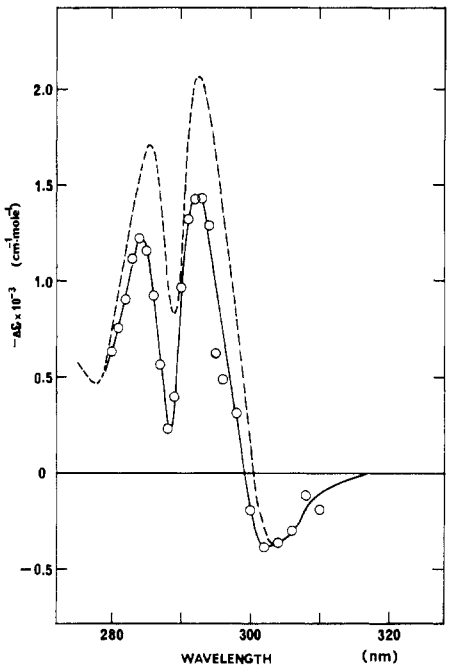

FIGURE 3: Difference absorption spectra for the alkaline and the acid conformational transitions at 0.5 M Gdn·HCl and 25.0 °C. The solid curve refers to the alkaline denaturation; open circles are calculated from the kinetic amplitudes observed by the pH jump from 11.6 to 8.0. The broken curve shows the equilibrium difference spectra for the acid denaturation at pH 2 (reference pH 6). Protein concentration and optical path length are the same as in Figure 2.

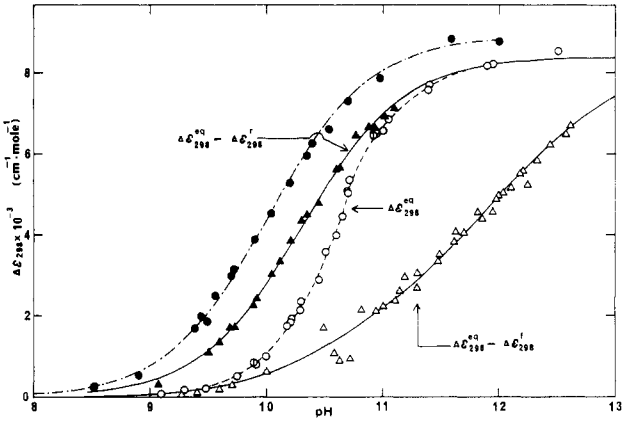

FIGURE 4: Spectrophotometric titration curves of tyrosines at 298 nm and 25.0 °C. (○, ⊙) The ionization curve under the equilibrium of the alkaline denaturation at 0.5 M Gdn·HCl (⊙: reversal); (△) the zero-time titration curve for the native protein at 0.5 M Gdn·HCl; (▲) the zero-time titration curve for the alkaline denatured protein at 0.5 M Gdn·HCl; and (●) the ionization curve for the protein completely denatured by 6.0 M Gdn·HCl. The reference solutions for $\Delta\epsilon^{eq}_{298}$ measurements are at pH 6 and contain the same concentrations of Gdn·HCl as the sample solutions. Two solid curves are theoretically drawn in terms of the pK values in Table I. Protein concentration and optical path length are the same as in Figure 2.

ticipate in the ionization equilibrium between pH 9.2 and 11.5. Thus, if the alkaline transition is a two-state reaction, $\Delta\epsilon^{eq}_{298}$ relates to the fractional extents of the native (N) and the alkaline (B) species, f_N and f_B, and to the degrees of tyrosine ionization in both the species, as shown in eq 2, where $\Delta\epsilon^0_{298}$

$$\Delta\epsilon_{298}{}^{eq} = \Delta\epsilon^0_{298}\sum_{i=1}^{n}(f_N\alpha_{N,i} + f_B\alpha_{B,i}) \qquad (2)$$

is the extinction coefficient for ionization of a single tyrosine residue, $\alpha_{N,i}$ and $\alpha_{B,i}$ are the degrees of ionization of the ith tyrosine residue in the N and the B states, respectively, and

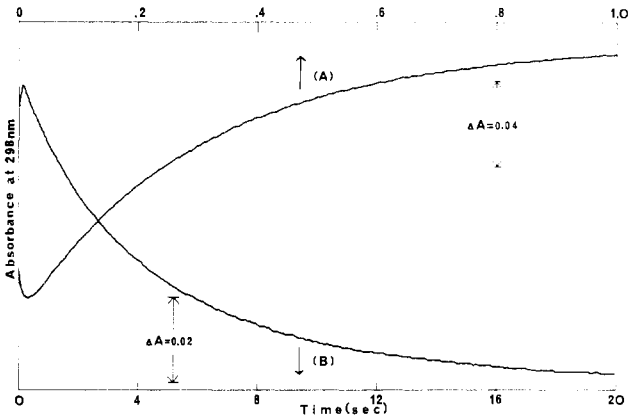

FIGURE 5: Typical time courses of the absorption changes at 298 nm after pH jump at 0.5 M Gdn·HCl and 25.0 °C: (A) forward pH jump from pH 5.5 to 11.3; and (B) reversed pH jump from 11.8 to 10.2. Protein concentration is ca. 0.05%.

n is the number of tyrosines contained (here, $n = 4$). From the two-state hypothesis

$$f_N + f_B = 1 \qquad (3)$$

Because of high ionic strength (≥0.6 M) used in this study, the ionizable groups can be regarded as titrating independently of each other, and $\alpha_{N,i}$ and $\alpha_{B,i}$ can be expressed in terms of the intrinsic dissociation constants, $K_{N,i}$ and $K_{B,i}$, as

$$\alpha_{N,i} = \frac{K_{N,i}}{K_{N,i} + a_{H^+}} \text{ and } \alpha_{B,i} = \frac{K_{B,i}}{K_{B,i} + a_{H^+}} \qquad (4)$$

where a_{H^+} is hydrogen ion activity.

Zero-Time Titration of Tyrosine Residues. When $\alpha_{N,i}$ differs significantly from $\alpha_{B,i}$ at the final pH in pH-jump experiments, the conformational change caused by the pH jump brings about the concomitant change in tyrosine ionization, and then such change can be observed as the time-dependent absorption change of $\delta A_{conf\text{-}ion}$ at 298 nm. The pH-jump experiments were performed in a manner that this absorption change observed will be useful for the purpose of estimating the zero-time titration curve of tyrosines in the purely N or the purely B state. The starting pH was set to pH 5.5~6 for the forward (raising pH) pH jump or to pH 11.7~11.8 for the reversed (lowering pH) pH jump, and the final pH was set to various pHs including the alkaline transition region; at each starting pH the protein is in the purely N or the purely B state (Figure 2). The typical runs are shown in Figure 5, and each of them is expressed as a single first-order process. The rate constant k of the process depends only on the final pH, and at pH >10 it increases remarkably with increasing pH; $k = 0.2\sim0.25$ s^{-1} at pH <10, $k = 500$ s^{-1} at pH 12.6, and detailed analysis on its pH dependence will soon be reported. The difference in extinction coefficients between the infinite and the zero times for the forward and the reversed pH-jump experiments, $\Delta\epsilon^f_{298}$ and $\Delta\epsilon^r_{298}$, can be evaluated from the kinetic amplitudes in both the experiments, and they are plotted against the final pH in Figure 6; the dead time correction was made for these kinetic amplitudes. The bell-shaped features of the plots in Figure 6 are expressed by the following relations:

$$\Delta\epsilon^f_{298} = \Delta\epsilon^0_{298}f_B\sum_{i=1}^{4}(\alpha_{B,i} - \alpha_{N,i}) \qquad (5)$$

and

$$\Delta\epsilon^r_{298} = \Delta\epsilon^0_{298}f_N\sum_{i=1}^{4}(\alpha_{N,i} - \alpha_{B,i}) \qquad (6)$$

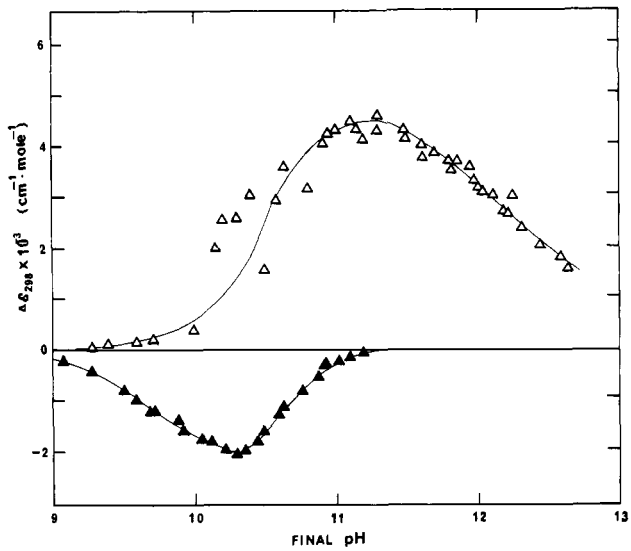

FIGURE 6: Dependence of $\Delta\epsilon^{f}_{298}$ and $\Delta\epsilon^{r}_{298}$ on final pH. (△) $\Delta\epsilon^{f}_{298}$; and (▲) $\Delta\epsilon^{r}_{298}$.

Table I: Comparison of the pK Values of Tyrosine Residues in α-Lactalbumin and Lysozyme

lysozyme		α-lactalbumin		
	pK_N[a]		pK_N	pK_B
Tyr-20	11.6	Tyr-18	11.8	10.3
Tyr-23	10			
		Tyr-36	11.8	10.3
Tyr-53	12.6	Tyr-50	12.7	10.3
		Tyr-103	10.5	10.3

[a] Tojo et al. (1966).

where f_N, f_B, $\alpha_{N,i}$, and $\alpha_{B,i}$ refer to the final pH. From eq 2, 3, 5, and 6, the following two equations are obtained:

$$\Delta\epsilon^{eq}_{298} - \Delta\epsilon^{f}_{298} = \Delta\epsilon^{0}_{298}\sum_{i=1}^{4}\alpha_{N,i} \quad (7)$$

and

$$\Delta\epsilon^{eq}_{298} - \Delta\epsilon^{r}_{298} = \Delta\epsilon^{0}_{298}\sum_{i=1}^{4}\alpha_{B,i} \quad (8)$$

These two quantities, which correspond to the zero-time difference extinction coefficients of tyrosine ionization after the pH jump but before the onset of the conformational transition, are also shown in Figure 4 as a function of pH, and these two sets of data represent the zero-time titration curves of tyrosines in the purely N and the purely B states, respectively. The solid curves in Figure 4 are theoretically drawn according to eq 4, 7, and 8 with the best-fit values of $K_{N,i}$ and $K_{B,i}$. The value of $\Delta\epsilon^{0}_{298}$ can be estimated from $\Delta\epsilon^{eq}_{298}$ at pH 12, where all the tyrosines become fully ionized because $\Delta\epsilon^{eq}_{298}$ of the protein completely denatured by 6 M Gdn·HCl is almost the same as that for 0.5 M Gdn·HCl at pH 12 (Figure 4). The resulting pKs are summarized in Table I together with the known values for hen egg white lysozyme (Tojo et al., 1966).

Discussion

The method presented in this paper is generally applicable to other proteins that contain tyrosines. In addition, even though the alkaline transition involves more than a single rate process or though it is irreversible, the slight modification of the analysis may make it possible to evaluate the zero-time titration curve of tyrosines in the native state. If all of the kinetic processes of the alkaline denaturation except tyrosine ionization are observable by the pH-jump method, the total kinetic amplitude for the forward process can be generally taken as a measure of the difference in ionization between the zero-time (native) and the final states.

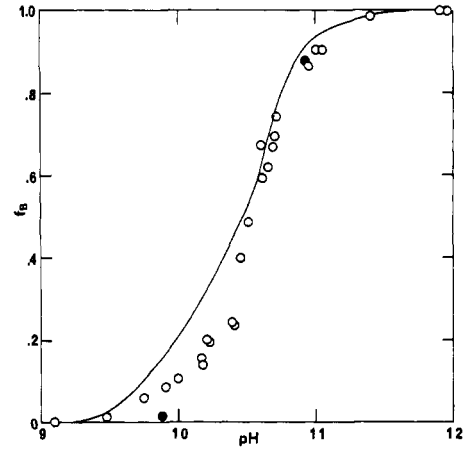

FIGURE 7: Normalized transition curves for the alkaline denaturation. The solid curve is calculated from the kinetic amplitudes, and circles represent the f_B values from Figure 2.

Validity of the Analysis. The present analysis is based on the assumption that there is no conformational transition other than a single first-order reaction observed. A support for this proposal is given by the comparison of the normalized equilibrium transition curve with that obtained from the kinetic amplitudes (Figure 7). The latter curve can be calculated from the relation that $f_B = \Delta\epsilon^{f}_{298}(\Delta\epsilon^{f}_{298} - \Delta\epsilon^{r}_{298})^{-1}$ and is shown in Figure 7. Both the curves derived from the different parameters for following the transition coincide with each other and this result suggests the validity of the analysis.

Environment of Tyrosines in α-Lactalbumin. Results in Table I reveal information about tyrosine residues in native α-lactalbumin. Out of the four tyrosines (Tyr-18, -36, -50, and -103), one is highly anomalous (pK = 12.7) and may be almost inaccessible to solvent in the native state. One of other residues ionizes normally (pK = 10.5), while the remaining two may be partly inaccessible (pK = 11.8). After the alkaline transconformation, all of them become titrated normally (pK = 10.3).

On the other hand, spectrophotometric titration studies on hen egg white and human lysozymes have shown that one of the tyrosines is very anomalous (Tojo et al., 1966; Latovitzki et al., 1971). The other two tyrosines in hen egg white lysozyme, accessible and partly accessible to solvent, have pK values of 10 and 11.6, respectively. X-ray structural and chemical modification data of hen egg lysozyme suggest that Tyr-*23* has the lowest pK, Tyr-*20* the intermediate pK, and Tyr-*53* the highest pK, which is hydrogen bonded and almost inaccessible to solvent (Blake et al., 1967; Strosberg et al., 1971; Kato & Murachi, 1971; Imoto et al., 1972; Allerhand et al., 1977).

The present results can be interpreted in terms of the conformational similarity of these proteins, and tentative assignment of tyrosines in α-lactalbumin is also shown in Table I. Two of the four tyrosines in α-lactalbumin are located in the corresponding positions in the hen egg white lysozyme sequence Tyr-18 (*20*) and -50 (*53*) (Brew et al., 1970), and these residues may have the pK values similar to those in lysozyme if both the proteins have similar stereoregular structures; italics refer to lysozyme. The less accessibility of Tyr-36 as compared with that of Tyr-103 is suggested by both the model building studies (Browne et al., 1969; Warme et al., 1974). Tyrosine-*38* in human lysozyme, which corresponds

to Tyr-36 in α-lactalbumin, may have a pK value close to 11.6 (Latovitzki et al., 1971).

One or two tyrosines in α-lactalbumin were known to be unreactive with cyanuric fluoride or with N-acetylimidazole, which may be consistent with the present results (Gorbunoff, 1967; Kronman et al., 1971). Prieels et al. (1975) have suggested that Tyr-103 in human α-lactalbumin, in which all the tyrosines occupy the same positions as in bovine α-lactalbumin, is more easily nitrated than Tyr-18 and that these two residues are more exposed than Tyr-36 and -50. The results are also consistent with the assignment in Table I.

Acknowledgments

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Conformations of Denatured and Renatured Ovotransferrin†

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ABSTRACT: Conformational properties of native, denatured, and renatured ovotransferrin were studied. The samples were denatured either in 7.2 M urea or in acidic (pH 3.0) conditions for periods up to a few hours. Combined data from quasi-elastic light scattering and transient electric birefringence were used to estimate the molecular dimensions under the various conditions. The native ovotransferrin is best described as a prolate ellipsoid with a major axis a = 68 Å and a minor axis b = 21 Å. Such an ellipsoidal shape is consistent with a globular particle where the solvation factor is ~0.28 mg/mg of solute. The urea-denatured sample was more expanded and more globular than the native sample. This observation was supported by a decrease in helical content, which was shown using circular dichroism data. Complete recovery of conformation and capacity to form a colored complex with Fe^{3+} seemed to occur with the simple dilution of urea or by adjustment of the low pH sample to pH 7.3.

Ovotransferrin (OT)[1] is an iron (Fe^{3+}) binding egg-white protein which is homologous with serum transferrin (Feeney & Komatsu, 1966; Greene & Feeney, 1968; Feeney & Allison, 1969; Osuga & Feeney, 1977; Thibodeau et al., 1978). The amino acid content of OT indicates there are 22 CySH residues (Osuga & Feeney, 1977), all of which exist as 11 disulfide bonds. These bonds are not easily destroyed even in the

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[1] Abbreviations used: OT, ovotransferrin; CD, circular dichroism; QELS, quasi-elastic light scattering; TEB, transient electric birefringence; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate; NTA, nitrilotriacetate.

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