Correlation Proton Magnetic Resonance Studies at 250 MHz of Bovine Pancreatic Ribonuclease. III. Mutual Electrostatic Interaction between Histidine Residues 12 and 119†

John L. Markley* and William R. Finkenstadt

ABSTRACT: The two adjacent active site histidine residues of bovine pancreatic ribonuclease A (histidine-12 and -119) yield proton magnetic resonance titration curves having Hill coefficients significantly less than unity (0.7 and 0.8, respectively). Three models postulating interactions with other titrating groups in the molecule have been used to approximate these anomalous experimental titration curves. Very good agreement with the data was obtained with models postulating mutual electrostatic interaction between histidine-12 and -119. The additional low pH perturbation of the chemical shift of the C(2)-H peak (but not the C(4)-H peak) of histidine-12 is attributed to a local conformational change with a pH_{mid} of about 3.5.

It is well established that histidine-12 and -119 of ribonuclease A (RNase A)[1] lie close to one another in the active site of the enzyme (for a review see Richards and Wyckoff, 1971). Rüterjans and Witzel (1969) first noted the deviations of the pH dependence of the nuclear magnetic resonance (NMR) chemical shifts of the C(2)-H peaks of His^{12} and His^{119} of bovine pancreatic ribonuclease A from normal titration curves. Since then abnormal NMR titration curves for these histidine residues have been observed in a number of laboratories (Cohen et al., 1970; King and Bradbury, 1971; Westmoreland and Matthews, 1973; Migchelsen and Beintema, 1973). The deviations are more pronounced at low ionic strength (Rüterjans and Witzel, 1969), and are absent in the presence of inhibitors such as cytidine 2′-monophosphate (Rüterjans and Witzel, 1969).

Rüterjans and Witzel originally proposed that the anomalies in the two NMR titration curves arise from a hydrogen bond between histidine residues 12 and 119. The X-ray crystallographic analyses of ribonucleases A and S (Kartha et al., 1967; Carlisle et al., 1974; Wyckoff et al., 1970), however, are not compatible with a direct hydrogen bond between the histidine residues. Two other mechanisms may account for the anomalous NMR titration curves: direct electrostatic interactions with other titrating groups, or indirect effects from more distant titrating groups mediated by a conformational change. Schechter et al. (1972) analyzed ^1H NMR titration data for His^{12} and His^{119} of RNase A according to a number of interactive models. They obtained an excellent fit to their data with a mutual interaction mechanism, but favored instead a second mechanism which gave a slightly better fit and which postulated interactions of the histidine residues with external groups.

The effects of adjacent charged groups on pK' values may be calculated on the basis of the Tanford-Kirkwood theory (Tanford and Kirkwood, 1957). Although this theory provides remarkably good results for small molecules (Tanford, 1957) it has serious limitations when applied to proteins (Tanford and Roxby, 1972). Nevertheless, one may use the theory [with the empirically derived parameters used to fit the lysozyme titration curve (Tanford and Roxby, 1972)] to estimate that a charged group within 12 Å of a given titrating group will perturb its pK' value by 0.1 pH unit or more (at ionic strengths between 0.1 and 1.0 m). Since pK' values of histidine rings in proteins may be determined by NMR titration studies with an accuracy of 0.1 pH unit or better, mutual electrostatic interaction between a histidine and another group having a similar pK' value lying within 12 Å should be detectable by NMR spectroscopy. Furthermore, it should be possible in principle to calculate the distance between the interacting groups if the appropriate parameters of the Tanford-Kirkwood equation are known.

We have reinvestigated the titration behavior of the active site histidine residues of RNase A by ^1H NMR spectroscopy at 250 MHz. The most satisfactory model tested assumes electrostatic interaction between His^{12} and His^{119} and a spectroscopic perturbation of the chemical shift of the C(2)-H peak of His^{12} at low pH. The calculated distance between the histidine residues based on the assumptions of Tanford and Roxby (1972) is in agreement with the X-ray results (Carlisle et al., 1974).

Experimental Section

Correlation ^1H NMR spectra at 250 MHz of RNase A samples were obtained as described in the first paper in this series (Markley, 1975a). The ^1H NMR data for the chemical shifts of the C(2)-H protons of His^{12} and His^{119} were analyzed according to four models.[2]

(1) Single noninteracting titration with a variable Hill coefficient n (Markley, 1973)

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[1] Abbreviations used are: RNase, bovine pancreatic ribonuclease; pH*, uncorrected pH meter reading of a D_2O solution made with a glass electrode standardized in H_2O buffers.

[2] In the following discussion formal charges on the species shown have been omitted for simplicity; H^+ is used instead of D^+ (the experiments were carried out in D_2O) for clarity and generality.

$$EH + \overset{K_i}{\rightleftharpoons} E + H^+ \quad (1)$$

where K_i is the proton dissociation constant of an individual histidine, and where for each dissociation

$$\frac{\delta_{low} - \delta_{obsd}}{\delta_{low} - \delta_{high}} = \frac{K_i^n}{K_i^n + [H^+]^n} \quad (2)$$

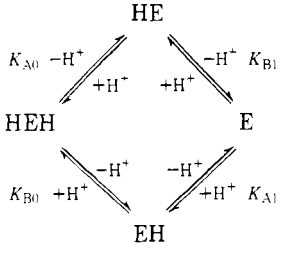
δ_{low} and δ_{high} are the chemical shifts of the histidine residue in its positively charged and neutral form, respectively. Least-squares analysis of the data to eq 2 was carried out with a fixed $n = 1$, and with a variable n.

(2) Interaction of each histidine with a second independent titrating group. The dissociation steps are

$$HEH \underset{+H^+}{\overset{K_{Xi}\ -H^+}{\rightleftharpoons}} EH \underset{+H^+}{\overset{K_i\ -H^+}{\rightleftharpoons}} E \quad (3)$$

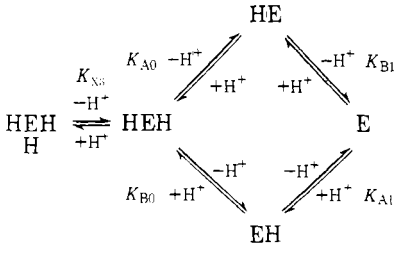
where K_i is the dissociation constant of a given histidine residue and K_{Xi} is the dissociation constant of an external group whose titration affects the chemical shift of the ith histidine NMR peak. This model is equivalent to "model 3" of Schechter et al. (1972) except that the chemical shifts of the intermediate forms δ_{HE} of curve H(2) and δ_{EH} of curve H(3) were fitted rather than assumed in the least-squares analysis.

(3) Mutual interaction of the two histidine residues. This is equivalent to "model 4" of Schechter et al. (1972). Both histidine titration curves are fitted simultaneously and four microscopic dissociation constants are obtained. In the analysis it was assumed that for both peaks H(2) and H(3): $(\delta_{HEH})_i = (\delta_{HE})_i$, and $(\delta_{EH})_i = (\delta_E)_i$.



(4)

(4) Mutual interaction of the two titrating histidines plus a spectroscopic perturbation of the chemical shift of peak H(3) in its acidic form at low pH.



(5)

This is essentially a combination of the two previous models as far as H(3) is concerned. Equations 6 and 7 give the pH dependence of the chemical shifts for peaks H(3) and H(2), respectively, for this model, where the dissociation constants (K's) have the same meaning as in models 2 and 3. δ_A and δ_B are the observed chemical shifts of peaks H(3) and H(2); $\delta_A{}^+$ and $\delta_B{}^+$ are the chemical shifts of the fully protonated histidines; $\Delta\delta_A$ and $\Delta\delta_B$ are the changes in chemical shift on deprotonation of each histidine; and $\Delta\delta_X$ is the spectroscopic change in the chemical shift of peaks H(3) at low pH.

The data for both sets of chemical shifts were fitted simultaneously for the nine parameters of eq 6 and 7. The intermediate chemical shift (δ_{HEH} of curve H(3)) was calculated from these parameters.

$$\delta_A = \delta_A{}^+ + \Delta\delta_A \frac{K_{A0}[H^+] + K_{A0}K_{B1}/[H^+]^2}{1 + (K_{B0} + K_{A0})/[H^+] + K_{A0}K_{B1}/[H^+]^2} + \Delta\delta_X \frac{K_{XA}}{K_{XA} + [H^+]} \quad (6)$$

$$\delta_B = \delta_B{}^+ + \Delta\delta_B \frac{K_{B0}/[H^+] + K_{A0}K_{B1}/[H^+]^2}{1 + (K_{B0} + K_{A0})/[H^+] + K_{A0}K_{B1}/[H^+]^2} \quad (7)$$

The nonlinear least-squares analysis was based on Deming (1964). The computer programs were written in Fortran and were run on a CDC 6600. The degree of fit to the data is given by the variance, the sum of the squares of the deviations normalized for the number of points and the degrees of freedom used in the fitting.

All chemical shifts are given as ppm from external 5% $(CH_3)_4Si$ in CCl_4. These may be converted to ppm from external $(CH_3)_4Si$ by the relation

$$\delta_{(CH_3)_4Si} = \delta_{5\%(CH_3)_4Si \text{ in } CCl_4} + 0.463 \quad (8)$$

Results and Discussion

The results obtained from analysis of the 250-MHz ^1H NMR data according to the four models are summarized in Table I. It is clear that the NMR titration curves for histidine-12 and -119 are not adequately described by simple titration curves: model 1, $n = 1$ (Table Ia). Much better agreement to the data was obtained when the Hill coefficients were also fitted (Table Ib). The experimentally derived Hill coefficients are 0.79 ± 0.03 and 0.67 ± 0.03 for peaks H(2) and H(3), respectively. Negative cooperativity of this kind is expected from electrostatic interactions between adjacent titrating groups having similar pK' values. The predominant groups involved could be the two histidines themselves since they have similar pK' values, or each histidine could interact with one or more other titrating groups. The latter explanation appears less likely since all the X-ray evidence shows the two histidine rings closer to one another than to adjacent ionizable groups (Kartha et al., 1967; Wyckoff et al., 1970; Carlisle et al., 1974). The model based on simple electrostatic interaction between the histidine residues (model 3, Table Ic) gives an excellent fit to the data except for the histidine C(2)-H peak H(3) at low pH. The titration shift ($\Delta\delta$) of H(3) is also abnormally large.

The fact that the C(4)-H peak from the same histidine residue (peak H(3')) has a normal titration shift and does not exhibit a parallel downfield shift at low pH (Markley, 1975a) suggests that the C(2)-H (peak H(3)) is affected by a local spectroscopic perturbation at low pH. Model 4 which incorporates this possibility yields a much better fit to the data (Table Id) and gives a more normal $\Delta\delta$ value. The experimentally derived pK' (or more properly pH_{mid}, since the transition is not assigned to a particular group) for the spectroscopic perturbation is 3.7 ± 0.2. The agreement between the titration curves calculated for this model and the experimental points is illustrated in Figure 1.

It is proposed above that a pH-induced conformational transition with a pH_{mid} of 3.7 ± 0.2 affects the chemical shift of peak H(3) but not that of peak H(3'). Supporting evidence for the existence of a conformational equilibrium

Table I: Least-Squares Fitting of 250-MHz NMR Titration Data for Active Site Residues Histidine-12 and Histidine-19 of Bovine Pancreatic Ribonuclease A to Four Models.

Peak (Assignment)[a]	pK′	Hill coeff n	Chemical Shift ppm from 5% $(CH_3)_4Si$ in CCl_4			Variance × 10^4
			δ_{low}	δ_{high}	$\Delta\delta$	
		a. Single pK, n = 1 (Model 1)				
H(2) (His^{119})	pK_B 6.19 ± 0.04	1.0[b]	−8.49 ± 0.01	−7.49 ± 0.02	1.00 ± 0.08	8.7
H(3) (His^{12})	pK_A 5.79 ± 0.07	1.0[b]	−8.66 ± 0.03	−7.43 ± 0.03	1.23 ± 0.06	3.1
		b. Single pK, n Fitted (Model 1)[c]				
H(2) (His^{119})	pK_B 6.19 ± 0.02	0.79 ± 0.03	−8.52 ± 0.01	−7.45 ± 0.01	1.07 ± 0.02	1.3
H(3) (His^{12})	pK_A 5.79 ± 0.02	0.67 ± 0.03	−8.73 ± 0.01	−7.36 ± 0.01	1.38 ± 0.02	1.8
		c. Mutual Interaction of the Two Histidines (Model 3)				
H(2) (His^{119})	pK_{B_0} 5.91 ± 0.04		−8.52 ± 0.01	−7.45 ± 0.02	1.07 ± 0.03	2.9
	pK_{B_1} 6.33 ± 0.04					
H(3) (His^{12})	pK_{A_0} 5.64 ± 0.03		−8.71 ± 0.01	−7.39 ± 0.02	1.32 ± 0.03	
	pK_{A_1} 6.06 ± 0.07					
		d. Mutual Interaction plus Spectroscopic Perturbation of Peak H3 (Model 4)				
H(2) (His^{119})	pK_{B_0} 6.01 ± 0.02		−8.51 ± 0.01	−7.46 ± 0.01	1.05 ± 0.02	
	pK_{B_1} 6.29 ± 0.02					
H(3) (His^{12})	pK_{A_0} 5.80 ± 0.03		−8.59 ± 0.02	−7.38 ± 0.01	1.21 ± 0.03	0.6
	pK_{A_1} 6.08 ± 0.04					
	pK_{XA} 3.74 ± 0.16		8.74 ± 0.01	−8.59 ± 0.02	0.15 ± 0.03	
		e. Interaction of Each Histidine with a Second Group (Model 2)				
H(2) (His^{119})	pK_B 6.40 ± 0.11		−8.20 ± 0.18	−7.46 ± 0.01	0.74 ± 0.19	1.1
	pK_{XB} 5.61 ± 0.24		−8.51 ± 0.01	−8.20 ± 0.18	0.31 ± 0.19	
H(3) (His^{12})	pK_A 6.01 ± 0.03		−8.47 ± 0.03	−7.39 ± 0.01	1.08 ± 0.04	2.8
	pK_{XA} 4.38 ± 0.16		−8.73 ± 0.01	−8.47 ± 0.03	0.26 ± 0.04	

[a] These assignments are according to Markley (1975a). [b] Assumed. [c] These data are reproduced from Markley (1975a) for comparison.

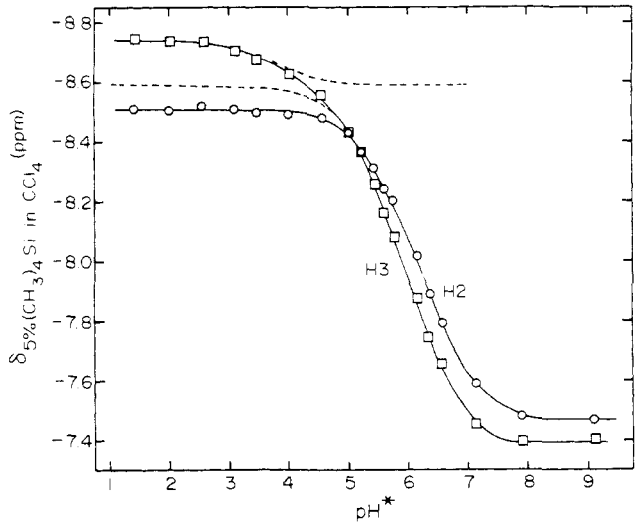


FIGURE 1: Chemical shift data for the C(2)-H NMR peaks of His^{12} (H(3)) and His^{119} (H(2)) [assignments of Markley, 1975a] fitted to model 4 (eq 5-7 in text). The model assumes mutual electrostatic interaction between the two histidine residues plus a spectroscopic perturbation of the chemical shift of His^{12} in its acidic form (shown as a dashed line).

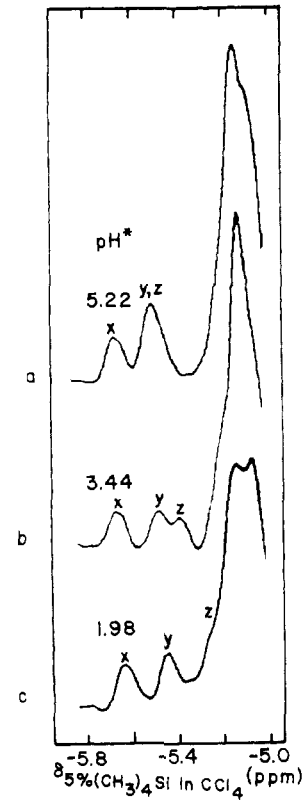


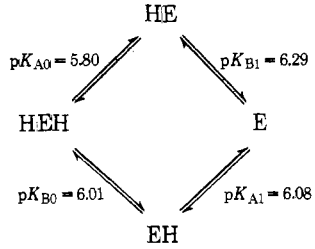
FIGURE 2: Proton magnetic resonance spectra of 250 MHz showing the three single-proton peaks (x, y, and z) tentatively assigned to backbone α-CH groups. (a) pH* 5.22; (b) pH* 3.44; (c) pH* 1.98.

in RNase A at this pH comes from studies of ^1H NMR peaks in the −5.7 to −5.2 ppm region between the aromatic envelope and the (residual) water peak. Three peaks (x, y, and z in Figure 2) of unit proton intensity have been resolved in this region. These peaks persist under all deuterium exchange procedures tried (Markley, 1975a) including reversible heat denaturation in D_2O at pH* 3. It is concluded that the peaks arise from carbon-bound protons, and they are tentatively assigned to abnormally deshielded α-CH groups on the protein backbone. Similar peaks have been found in spectra of a variety of other proteins (J. L. Markley, unpublished). Double resonance experiments have demonstrated spin-spin coupling between two such reso-

nances in spectra of bovine pancreatic trypsin inhibitor (Kunitz) and peptide N–H resonances indicating that the former correspond to α-CH groups (R. Rowan and J. L. Markley, unpublished).

Peaks x, y, and z of RNase A are sensitive to protein conformation and shift upfield as RNase A begins to unfold at low pH (Figure 3). The direction of these shifts is in agreement with the assignment of peaks x, y, and z to α-CH rather than aromatic side chain groups. Conformational transitions in RNase A below pH 3 have been characterized by optical spectroscopy (Hermans and Scheraga, 1961; Bigelow and Krenitsky, 1964). Peak 2 is affected by a second transition with a pH_{mid} of 3.46 ± 0.10. The pH_{mid} of this transition agrees within experimental error with that postulated to affect the chemical shift of histidine peak H(3).

The microscopic dissociation constants for the active site histidines obtained from the fourth model are:



(9)

The ΔpK resulting from mutual interaction between His^{12} and His^{119} is

$$(pK_{A1} - pK_{A0}) = (pK_{B1} - pK_{B0}) = 0.28 \pm 0.05$$

At an ionic strength of 0.3 *m*, 0.3 pH unit corresponds to a distance of 7.7 ± 0.8 Å between the two histidine rings according to the analysis of Tanford and Roxby (1972). The calculation assumes an internal dielectric constant of 4.0 and a depth of charge parameter *d* of 0.4 Å. This depth of charge value has no theoretical basis but was the empirical value found to yield the most consistent results for the potentiometric titration curve of lysozyme (Tanford and Roxby, 1972).

The calculated distance is in remarkable agreement with the distance of 7 Å found between the rings of His^{12} and His^{119} by X-ray diffraction analysis of uninhibited RNase A crystals (Carlisle et al., 1974). Distances calculated from ΔpK data by the Tanford–Kirkwood theory are strongly dependent on the value used for the depth of charge, *d* (Tanford and Roxby, 1972). In light of the arbitrary nature of this parameter, the agreement with X-ray data may be fortuitous. NMR spectroscopy, however, provides one of the few ways of testing the validity of the Tanford–Kirkwood theory for individual groups in proteins, and future studies of this kind should be of interest.

While mutual electrostatic interaction of the two active site histidine residues provides an attractive explanation for the anomalous NMR titration curves, other possible mechanisms cannot be ruled out entirely. Schechter et al. (1972) preferred a model involving external titrating groups. If such a mechanism is to be predominant, either the histidine residues of RNase A in solution must be farther apart than in crystals or there must be a serious error in the empirical depth of charge parameter used to calculate the distance (Tanford and Roxby, 1972).

The model favored by Schechter et al. (1972) (model 2) does give an adequate agreement to our data (Table Ie). The simple model requires the existence of two external groups having pK' values of 5.6 ± 0.2 (affecting H(2)) and 4.4 ± 0.2 (affecting H(3)). If the low pH spectroscopic perturbation of H(3) is added to this model the fit to curve H(3) becomes slightly better, and the three pK' values affecting H(3) are 3.5 ± 0.3, 5.3 ± 0.3, and 6.2 ± 0.1. The external perturbations affecting H(2) and H(3) have closer pK' values (5.6 ± 0.2 and 5.3 ± 0.3) under this model and could arise from a single transition. A conformational transition does occur in RNase A at this pH, namely the transition having a pH_{mid} of 5.6 which affects His^{48} and a tyrosine residue and has been ascribed to ionization of Asp^{14} (Markley, 1975b). There is evidence that inhibitor binding at the active site changes this conformational equilibrium (Hammes and Walz, 1969; Markley, 1975b). Therefore, it is plausible that the conformational equilibrium may affect the chemical shifts of the active site histidine residues.

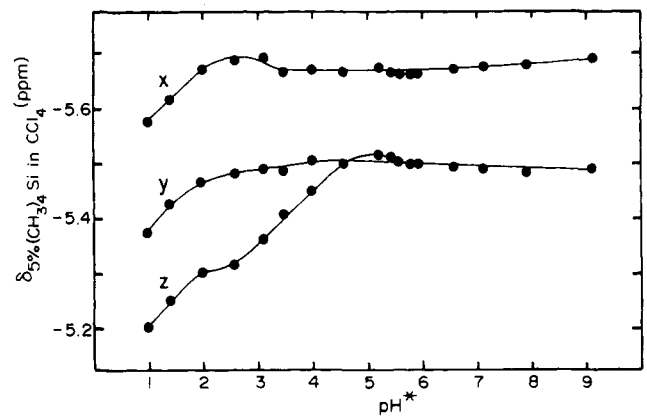


FIGURE 3: The pH dependence of peaks x, y, and z tentatively assigned to backbone α-CH groups. All three peaks shift upfield below pH* 3. Peak z shows an additional transition with a pH_{mid} 3.46 ± 0.10.

In deciding between these two mechanisms the principle of parsimony must be followed. The mechanism involving mutual electrostatic interaction is consistent with the known proximity of the imidazole rings of His^{12} and His^{119}; it provides a ready explanation for the ionic strength dependence of the pK' values; and it does not require the existence of any external interacting groups, although the assumption of a spectroscopic perturbation of peak H(3) at low pH improves the agreement with the data.

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Limited Hydrolysis of Bovine Plasma Albumin at Neutral and Alkaline pH Catalyzed by Associated Proteinases†

Koichiro Aoki‡ and Joseph F. Foster*

ABSTRACT: Proteinase contaminants in some plasma albumin samples have previously been shown to produce cleavage of the albumin molecule at acid pH. The F conformer, existing at pH 3.8, is cleaved near residue number 400 to yield a large N-terminal fragment of approximately 46,000 daltons. No cleavage was found at pH above approximately 4.4. It is shown in this paper that the proteinase contaminants are active over a broad pH range from 2.5 to 11.4 provided conditions are such as to induce some breakdown of the native conformation of the albumin molecule. Addition of Tris–borate buffer (0.1 *M*) at pH 7.5–9.7 is sufficient to permit cleavage. At pH near 9 this occurs predominantly near residue 230 to yield two fragments of approximately 42,000 and 27,000 daltons. Near neutral pH substantial cleavage occurs in 4–8 *M* urea solution or in the presence of sodium dodecyl sulfate (AD_{110} complex). Under these conditions there are two large fragments (42,000 and 47,000 daltons) and essentially two small ones (20,000–27,000 daltons). Under conditions where there is no cleavage at 38–40°, substantial cleavage results at 50–65° but enzyme inactivation also occurs toward the top of this range. The alkaline activity is inhibited by soybean trypsin inhibitor but not by pepstatin; the reverse is true of the low pH activity. Cleavage at neutral or alkaline pH under the various conditions occurs primarily at X–Leu bonds while the low pH activity was already shown to occur at X–Phe. These facts suggest the presence of at least two enzymes. There is surprisingly little pH dependence over the range 7.5–9 in any of the media examined, even though albumin is known to undergo a significant conformational change in this range, the N → B transition. This transition is thought to be essentially a tertiary change with little loss of helix content. It is suggested that loss of native secondary structure, especially uncoiling of helical regions, is crucial to permit attack by these enzymes.

Wilson and Foster (1971) made the chance observation in disc acrylamide gel electrophoresis patterns that certain albumin preparations undergo limited proteolytic cleavage when exposed to pH below 4.3 for prolonged periods. They concluded that the enzyme responsible has no activity on the N form of the protein, but specifically cleaves the F form, existing at pH near 3.8, in one region. The initial cleavage yields two peptide chains of molecular weight approximately 46,000 and 24,000 which are disulfide linked and represent respectively the amino-terminal and carboxyl-terminal portions of the parent peptide chain. A subsequent cleavage removes approximately 30 additional amino acid residues from the smaller peptide. At lower pH, where molecular expansion of the albumin molecule is known to occur, more extensive degradation was observed.

The results thus indicated a remarkable dependence of the mode and degree of cleavage on the conformational state of the albumin substrate. From this point of view, it was somewhat surprising that no evidence of cleavage was seen above pH approximately 4.4 and up to pH 9 since the albumin molecule is known to undergo a conformational change in the neutral to alkaline pH range (Leonard et al., 1963; Harmsen et al., 1971; Zurawski and Foster, 1974) which has at least some superficial resemblance to the N–F transition.

Recently one of us (Aoki and Nagaoka, 1973) showed that Tris–borate–EDTA buffer systems provoke a small conformational change in the albumin molecule at pH 9. As the concentration of Tris buffer is increased from 0 to 0.1 *M* at substantially constant ionic strength there is an approximate 10% drop in $s_{20,w}$. In parallel, optical rotatory dispersion (ORD) studies indicated an approximate 5% decrease in the content of α helix. This prompted us to seek evidence of proteolytic degradation in Tris–borate buffer systems at pH 9. A relatively specific cleavage was found which, however, differs in position from that obtained with

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‡ Permanent address: Department of Synthetic Chemistry, Gifu University, Kagamigahara, Gifu Pref. 504, Japan.