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Folding of Staphylococcal Nuclease: Magnetic Resonance and Fluorescence Studies of Individual Residues

(histidine/protein structure/acid denaturation/proton/spectroscopy)

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ABSTRACT The reversible unfolding and folding of staphylococcal nuclease in the acid transition has been studied by 220 MHz proton magnetic resonance spectroscopy. The values of area, line-width, and chemical shift of each of the imidazole C2 proton resonances of the four histidine residues have been measured in this transition. The change of areas of three histidine resonances and the change of fluorescence of the single tryptophan residue, as a function of pH, appear to follow a single equilibrium. In contrast, a fourth histidine resonance follows a biphasic transition. These findings indicate that local conformational changes can be detected by magnetic resonance spectroscopy in the cooperative transition of the overall structure.

The information for the folding of proteins to their biologically active conformation is contained within the linear amino-acid sequence of the polypeptide chain (1). Thermodynamic and kinetic studies of the denaturation and renaturation of proteins constitute an important approach towards understanding the relationship between primary sequence and three-dimensional structure (2, 3).

High-resolution proton magnetic resonance (PMR) provides detailed information about the environment of individual amino-acid side chains during conformational equilibria (4, 5). We report here a study using the discrete imidazole C2 proton resonances in the 220 MHz PMR spectrum of staphylococcal nuclease (EC 3.1.4.7) (6) to follow the reversible unfolding and folding at acid pH. Equilibrium and kinetic aspects of this conformational transition have been studied by other techniques (7–10) for this protein, whose amino-acid sequence (11) and three-dimensional structure (12) are known. Thus, the four histidine residues spatially defined by these chemical and physical studies may serve as probes of microscopic conformation during acid denaturation. Our results show that the PMR spectral changes of the resonance of one of these histidine residues when perturbed by acid is not described by the single cooperative transition that describes the changes in the resonances of the other histidine residues and the change in the fluorescence of tryptophan-140.

MATERIALS AND METHODS

Staphylococcal nuclease was prepared by methods previously described (13). Samples were lyophilized several times from D_2O (99.7% three times, then 100% twice; Aldrich Chemical Co.) and adjusted to 100 mg/1.0 ml of solution in 0.1 M NaCl by the addition of 100% D_2O and NaCl. The molar ratio of enzyme and calcium ions in these preparations was found by elemental analysis to be 2:1. All pH measurements were performed in the PMR cell with a long, thin combination electrode (180 × 3 mm; Instrumentation Labs.) and a Radiometer model 26 pH meter. pH was adjusted in the PMR cell by the use of micro-pipettes (Cole-Parmer) fitted with Teflon needles (Chemical Rubber Co.) and containing DCl and NaOD (Merck of Canada) solutions (both 0.1 and 1.0 M in acid or base; also 0.1 M in NaCl). Quoted values of pH are uncorrected meter readings.

Spectra were recorded on a Varian Associates HR-220 spectrometer with a helium-cooled superconducting magnet at the ambient probe temperature of 22 ± 1°C. A range of 250 Hz was used with a sweep rate of 1 Hz/sec. A Varian C1024 time-averaging computer was used that was triggered on a side-band of HDO in the sample. Each spectrum was calibrated directly with the same 6% tetramethylsilane in CCl_4 sample by the sideband technique. All chemical shifts are quoted in ppm downfield from this standard. The 220 MHz spectrometer was used continuously for any one series of experiments in order to minimize variation due to magnetic field drift and other instrumental adjustments. Spectra were digitized by the CALMA (California Computer Products, Inc.) scanner, and curve-fitting was performed with the "MODELAIDE" program as described (14). Computer-calculated results were drawn by a CALCOMP (California Computer Products, Inc.) plotter.

For determinations of relative tryptophan emission intensity, an Aminco-Bowman (American Instrument Co.) spectrophotofluorometer was used. The fluorometer contained an RCA 1P28 photomultiplier tube and a grating blazed at 300 nm.

RESULTS

PMR spectra

The resonances of imidazole C2 protons appear in a region of the PMR spectrum of staphylococcal nuclease downfield from the main aromatic side-chain resonances (15). As shown in Fig. 1, these discrete resonances may be followed through a range of pH values. At pH 5.03, the protein is native by all the criteria studied (7), and the four proton resonances (H-1–H-4) represent the four histidine residues (6) and appear to have equal areas. Resonance H-3 has been assigned to histidine-124, because of spectral differences between the Foggi and V8 enzymes (15). Resonance H-2 is thought to represent histidine-46, the histidine residue nearest the active site (12), because of the effects of ligands and limited tryptic digestion (15) and of Eu^{3+} ions (unpublished results) on its titration

curve. During the acidification of staphylococcal nuclease, the four imidazole C2 proton resonances coalesce into the single resonance of histidine in denatured protein, which resonance occurs at about the same chemical shift value as H-2.

In order to test for concentration-dependent effects, such as aggregation, spectra were recorded at two lower protein concentrations (11 and 33 mg/ml) for the native (pH 5.0) and denatured (pH 3.6) protein. The line-widths of the individual resonances and their chemical shift values showed no significant deviations from the values at 100 mg/ml.

Analysis of PMR spectral changes during denaturation

The PMR spectra shown in Fig. 1 were quantitatively analyzed by fitting them to Lorentzian functions with the aid of a digital computer (Fig. 2) (14). Three parameters related to each resonance were calculated: area, line-width (resonance width at half-height), and chemical shift (midpoint of resonance in a scale related to an external standard). The area of each resonance is the parameter that represents concentration of protons in that particular environment. The chemical shift of each resonance describes the electromagnetic properties of the environment. The line-width can yield information on hydrodynamic changes and exchange between chemical environments.

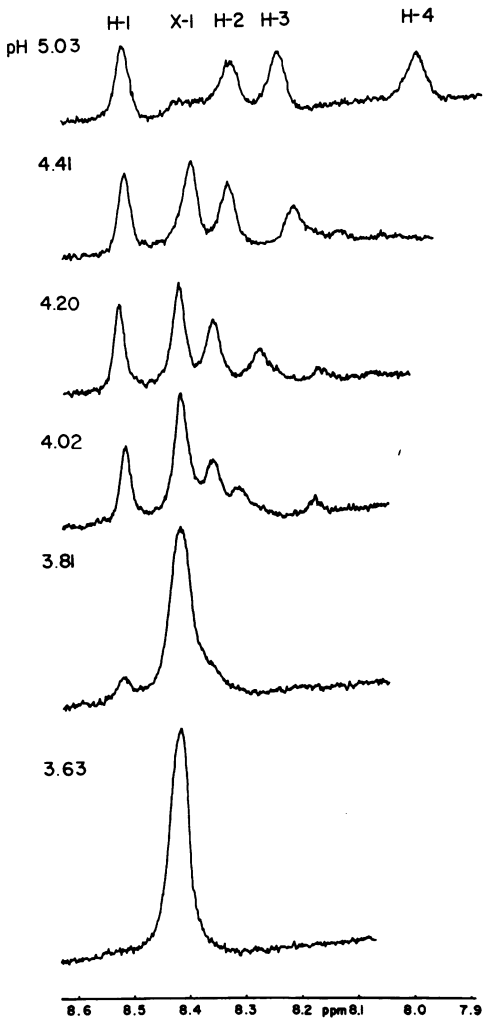

FIG. 1. PMR spectra of staphylococcal nuclease denatured by the successive addition of small amounts of DCl. Each spectrum is the result of 100 time-averaged scans. H-1 to H-4 are the imidazole C2 proton resonances. In the pH 5.03 spectrum, a small resonance, X-1, that does not titrate, is observed at 8.43 ppm and is presumed not to originate from histidine (6). At lower pH, a second small resonance of undetermined origin is observed at 8.15–8.20 ppm. Its appearance may be related to the denaturation transition.

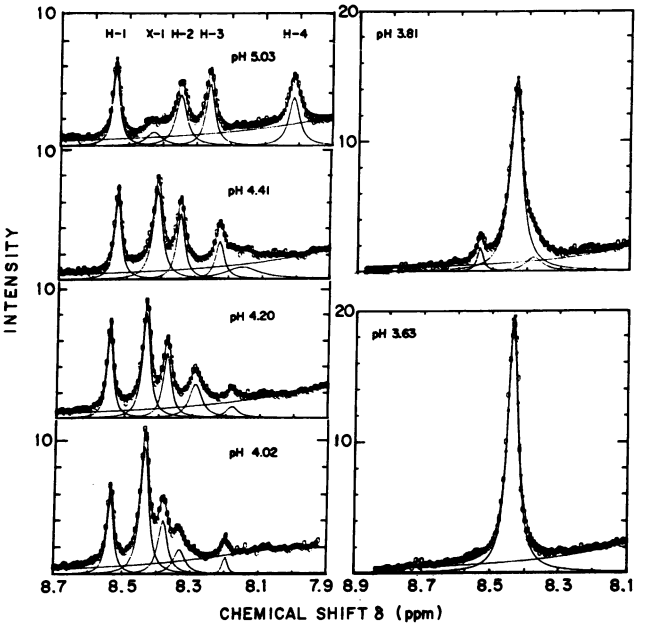

FIG. 2. Computer curve fits to Lorentzian functions of the PMR spectra shown in Fig. 1. *Open circles* and *asterisks* represent the experimental and calculated points, respectively. *Continuous lines* represent the theoretical component curves. The arbitrary scale of the ordinate is identical for all spectra.

At pH 5.03, the four areas are equal, in confirmation of previous studies (6). The total area of the histidine resonances remains essentially constant (mean ± 10%) throughout the acid transition. Fig. 3 shows that resonances H-1, H-3, and H-4 decrease in area, while the resonance about H-2 increases in area. The midpoint of the equilibrium of H-4 with the denatured environment occurs at a higher pH (overall half-change at pH 4.1) than the equilibria with H-1 and H-3 (half-change at pH 3.9). At pH 4.1, resonance H-4 has lost 40 to 50% of its area, while H-1 and H-3 still have unit area values. These differences in areas suggest that the environments of the individual histidine residues have different stabilities with respect to acid perturbation. The acid-induced transitions appear steeper than simple proton dissociation curves, which suggests that these conformational equilibria involve cooperative interactions.

Chemical shift values, from a number of acid denaturation experiments, were plotted as a function of pH (Fig. 4). These titration curves are described by the simple proton equilibria with the same pK values as those determined for the histidine residues of the native enzyme (6). Thus, the changes in spectral position observed for each of the imidazole C2 proton resonances are not related to the denaturation process, but only to the protonation of the imidazole nitrogen atoms. This also indicates that the observed changes in areas of these resonances are not caused directly by titration of

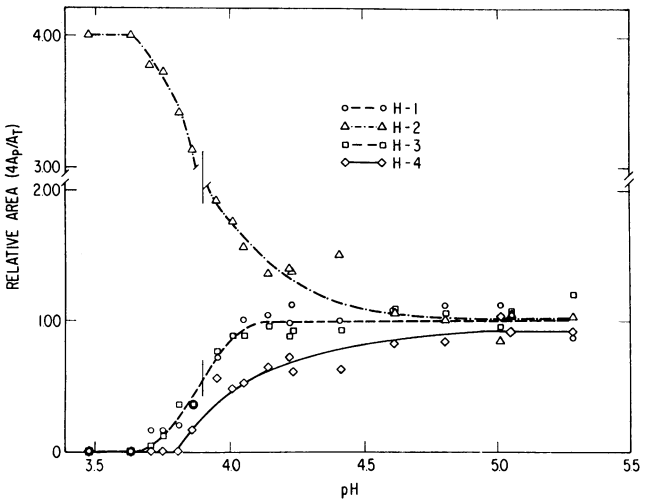

FIG. 3. The areas of the imidazole C2 proton resonances, from two series of spectra, as a function of pH. The calculated area value for each resonance (A_p) is expressed as a fraction of the total area of the imidazole C2 proton resonances (A_T) normalized to 4.0 at each pH, since for all pH values the total area of these resonances remained constant. The curves are hand-drawn approximations to the data. Vertical lines at pH 3.9 represent the pH of half-change of resonances H-1, H-3, and that about H-2.

nearby amino-acid side chains or by ion binding. The chemical shift of the resonance of the C2 protons in nuclease at pH 3.6 is identical to their chemical shift in 8 M urea, indicating that the average electronic environments of the histidine residues in the two denatured species are similar.

Since separate resonances are observed for the native and denatured forms at intermediate pH values, a slow conformational transition is occurring during acid denaturation (5). The separation in chemical shift between resonance H-1 and the denatured resonance indicates that the exchange time for these two resonances must be significantly greater than 14 msec, which is consistent with rates determined by other methods (9, 10).

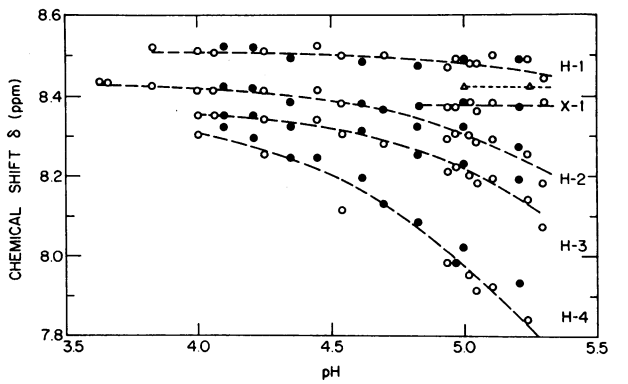

FIG. 4. Chemical shift values of observed resonances as a function of pH. *Open* and *closed circles* represent the chemical shifts of individual resonances after additions of acid and base, respectively. *Open triangles* represent the chemical shift of nuclease in 8 M urea. *Dashed lines* represent Henderson-Hasselbalch functions for each histidine titration curve. In the native enzyme, the pK values of H-1, H-2, H-3, and H-4 are 6.50, 5.74, 5.71, and 5.37, respectively, in 0.1 M NaCl (6).

In contrast to the parameters of area and chemical shift, the line-widths of the individual resonances, when plotted as a function of pH, do not fit any simple relationship.

Reversibility of PMR spectral changes

PMR spectroscopy requires protein solutions of greater than 1 mM to be scanned at constant temperature over a period of several days. In order to determine whether the folding and unfolding processes of nuclease are reversible under these conditions, the renaturation of a denatured sample was studied. Staphylococcal nuclease at pH 5.07 was denatured by lowering the pH to 3.57, where only the single resonance of denatured protein was observed (Fig. 5). Elevation of the pH yielded, successively, spectra qualitatively similar to those spectra (Fig. 1) obtained at intermediate pH values in the unfolding direction. At pH 5.03, a spectrum similar to the original was obtained.

A second PMR criterion of reversibility is the identity of the proton equilibrium functions of the change in chemical shift when measured either by decreasing or increasing pH. As shown in Fig. 4, the individual imidazole nitrogen atoms retain their distinct affinities for protons after denaturation and renaturation.

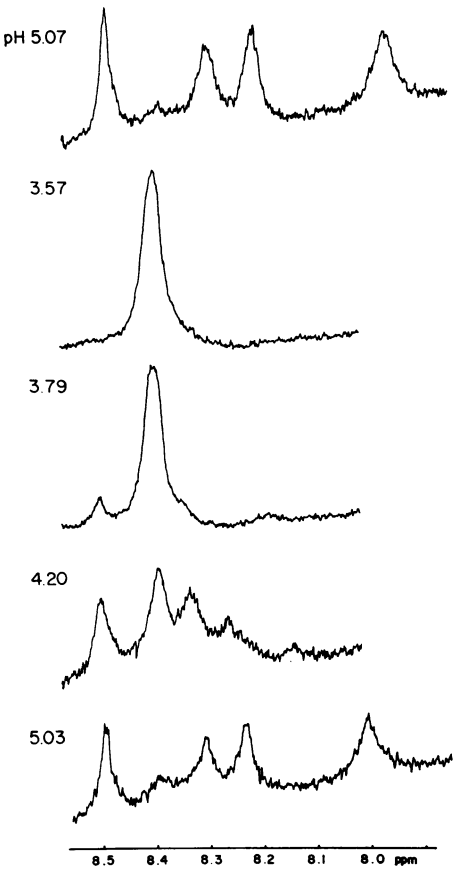

FIG. 5. PMR spectra of staphylococcal nuclease during denaturation and renaturation. The protein was denatured by the addition of DCl and renatured by the addition of NaOD. Each spectrum is the result of 50 time-averaged scans. The scales of the ordinates are not identical for all spectra. In the spectrum of renatured nuclease at pH 5.03, the increased prominence of X-1 may represent a small quantity of denatured material. An increase in the upfield water resonance with time leads to an elevation of the baseline.

Comparison of PMR with fluorescence changes

Nuclease has a single tryptophan residue, the fluorescence of which is sensitive to the acid transition (7). A sample of nuclease in D_2O was denatured by the addition of NaOD and renatured by the addition of DCl (Fig. 6). The fluorometric titration curves in each direction are superimposable and very similar to those of ordinary nuclease in aqueous solutions.

This result suggests that this cooperative transition in acid is not affected by the substitution of D_2O for water. This contrasts, for example, with the thermal transitions of poly(L-glutamate) and ribonuclease A (16).

The half-change of the tryptophan emission occurs at a similar pH value (3.8–3.9) to the half-change in area of the resonances H-1, H-2, and H-3 (3.9). As in water (10), there is a slight asymmetry in the tryptophan fluorescence change above pH 4. This small perturbation could possibly represent an additional structural transition which affects the tryptophan environment and that may be related to the separate equilibrium of resonance H-4.

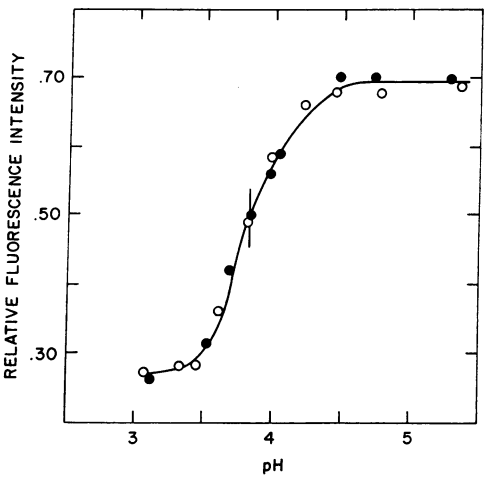

FIG. 6. The fluorescence of tryptophan-140 as a function of pH in D_2O. *Closed* and *open circles* represent successive additions of DCl and NaOD, respectively. Fluorescence emission was measured at 350 nm, with excitation at 290 nm.

DISCUSSION

PMR spectroscopy has been used by several research groups to study the process of protein denaturation (17–22). These studies demonstrated that resonances sharpen in going from the native to the denatured state because of the magnetic equivalence of chemically identical protons in the disordered form and because of the increased flexibility. The conformational transitions of bovine pancreatic ribonuclease A (EC 2.7.7.16, ref. 20) and of (hen) egg-white lysozyme (EC 3.2.1.17, ref. 21) have been studied at 220 MHz. In the RNase study, the weighted average of the chemical shifts of resonances in the aromatic region was calculated, while in the lysozyme study, areas of several types of resonance were measured. In both cases, the transitions were interpreted as being consistent with a *single cooperative* equilibrium. PMR studies, at 100 MHz, of individual tyrosine resonances of staphylococcal nuclease during alkaline denaturation have been described (15). Differences among the pH values of half-change of the transitions in chemical shifts of each of these residues were interpreted as evidence for *distinct* conformational equilibria. The denaturization of ribonuclease by formic acid and of lysozyme by urea, as followed by peak intensity at 60 MHz, have been interpreted in terms of *multiple-step* processes (22).

The PMR spectral region containing the imidazole C2 protons from the four histidine residues of staphylococcal nuclease constitute a "window" (23) through which detailed changes during conformational transitions may be observed. In previous experiments, with a 100 MHz spectrometer, it was not possible to obtain quantitative data for the imidazole C2 resonance areas (unpublished results). The very high resolution and sensitivity of the 220 MHz spectrometer markedly facilitates quantitative analyses. Accurate determinations of the areas, chemical shifts, and line-widths of these individual resonances may now be made by use of a digital computer to fit a series of Lorentzian functions to the spectra (14).

The change in the environments of resonances H-1 and H-3, as observed from the area measurements, follows a *strongly cooperative* function with a half-change at pH 3.9. The area change of resonance H-4 is complex and has an overall half-change at pH 4.1. The increasing area at H-2, with an overall half-change at pH 3.9, reflects the composite change of all the histidine residues. The complex relationship between the area of the H-4 resonance and pH suggests that a local conformational change involving this histidine residue is being observed during progressive acidification.

The fluorescence of the single tryptophan of staphylococcal nuclease has been studied in D_2O solutions as a function of pH. This parameter also follows a reversible, cooperative transition with a half-change at pH 3.8–3.9. Fluorescence emission and PMR parameters are sensitive to the spatial relationships of nearby dipolar groups. The changes of these parameters due to additions of acid and base may be directly related to perturbations of the specific chemical environments within the protein structure of the tryptophan and histidine microscopic probes. Reduced viscosity and molar ellipticity at 220 nm of nuclease, as a function of pH, also have half-change values at about pH 3.9 (unpublished results). Thus, the change in hydrodynamic properties, tryptophan emission, and the areas of H-1, H-2, and H-3 probably represents the major structural rearrangement in nuclease folding. The area of resonance H-4, however, seems to reflect some *local structural change* that occurs at slightly more alkaline pH.

Kinetic studies of the change in tryptophan fluorescence emission have indicated the presence of at least two processes in the renaturation of acidified enzyme (9). Temperature-dependence studies suggest that these rate processes may correspond to a nucleation of ordered structure followed by the formation of hydrophobic interactions (10). In equilibrium studies, the asymmetry of both tryptophan and tyrosine fluorescence emission as a function of pH also suggests the presence of a perturbation in the pH range of 4–5 (7, 10).

The occurrence of these conformational transitions suggests that the folding of this enzyme involves *detectable* intermediates in addition to the principal, cooperative conformational transition of the entire macromolecule. These intermediates may contribute to the sequence of events leading to the formation of the biologically active enzyme. Such microscopic structural changes during folding may also be related to the conformational motility observed in nuclease that is in solution (24).

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