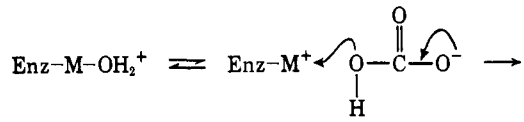
tivity not unlike that of bicarbonate, so that there appears to be little catalytic advantage to this pathway. On the other hand, taking together the indications that catalysis by HOBr proceeds in a concerted mechanism, and evidence—the reasonable calculated Brønsted slope—that catalysis by $H_2PO_4^-$ and $H_3O^+$ proceeds similarly, it appears that there is some special propensity for a concerted decarboxylation with bicarbonate. We suggest the outline of such a mechanism for carbonic anhydrase in eq 15. This mechanism does not circumvent the dilemma, outlined above, concerning the insufficient rate of proton transfer.



$$\text{Enz—M—OH} + CO_2 \quad (15)$$

# Nuclear Magnetic Resonance Study of the Mechanism of Reversible Denaturation of Lysozyme

**C. C. McDonald,* W. D. Phillips, and J. D. Glickson**

*Contribution No. 1553 from the Central Research Department, Experimental Station, E. I. du Pont de Nemours and Company, Wilmington, Delaware 19898.* *Received May 13, 1970*

**Abstract:** Reversible denaturation of hen egg white lysozyme was studied by proton magnetic resonance spectroscopy. Temperature, pH, and guanidine concentration were employed as denaturation variables. Denaturation and renaturation appear by this technique to be cooperative transitions between a native state N and a denatured state D. Through the transition region of the denaturant parameter the process is described by the equilibrium $N \rightleftarrows D$; no transition intermediates were detected. In the thermal transition at pH 3.3 between 60 and 75°, $\Delta H$ = 73.5 kcal mol$^{-1}$, $\Delta S$ = 215 cal deg$^{-1}$ mol$^{-1}$, and the $N \rightleftarrows D$ interconversion rate lies between $1.4 \times 10^{-3}$ and $2 \times 10^2$ sec$^{-1}$. The pmr spectra indicate that D is extended in solution so that all residue side chains are solvated (but does not exclude the possibility of some residual secondary structure) and that N is a unique conformation except for possible mobility of surface residue side chains.

Biological activities of proteins usually are associated with the folded structures that they assume under physiological conditions. The three-dimensional structures of a number of proteins in the solid state now have been determined by X-ray crystallography, including that of hen egg white (HEW) lysozyme,[1] the subject of this study. Many questions remain, however, regarding the structures of proteins in solution, their interactions with the environment, and the processes of protein folding and denaturation.

Several investigations of the denaturation of HEW lysozyme in aqueous solution by optical techniques[2-7] have been reported. The compact folded conformation of the protein converts to a cross-linked (four residual disulfide bonds), random-coil state upon heating, lowering the pH, or addition of guanidine hydrochloride. On reversal of the denaturant parameter, substantial or complete renaturation of the protein to the native state is achieved. The processes of unfolding and folding are rapid for most of the denaturation–renaturation conditions that have been studied so that equilibrium is achieved between the native and denatured states in times from a few seconds to several minutes. If procedures are used during denaturation that break the disulfide bonds, renaturation can occur but at a much slower rate.[8]

Sophianopoulos and Weiss[2] concluded that thermal denaturation of lysozyme proceeded without stable intermediates by a two-state process, where N and D

$$N \rightleftharpoons D$$

are, respectively, the native and denatured (cross-linked random-coil) states. Tanford and coworkers concurred in this conclusion for denaturation induced by guanidine hydrochloride but stated that the final state produced by thermal denaturation retains elements of structure beyond just cross-linking of the random-coil peptide chain by disulfide bonds. They observed changes in the optical rotation of thermally denatured lysozyme upon addition of guanidine hydrochloride that were interpreted in terms of folded structure which was dissipated by the chemical denaturant.[5] The denaturation of lysozyme was represented as

$$N \rightleftharpoons X \rightleftharpoons D$$

where D represents the truly denatured state reached only in concentrated guanidine hydrochloride and X is a distinct but only partially denatured state, structured in undefined fashion, attained by strictly thermal denaturation.

* Address correspondence to this author.

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Previous studies of aqueous solutions of HEW lysozyme by proton magnetic resonance (pmr) spectroscopy[9-14] have established that the structural alterations associated with denaturation of the protein are accompanied by marked changes in the pmr spectrum. Sternlicht and Wilson[10] showed that manifestations of thermal denaturation of lysozyme observed by optical rotatory dispersion paralleled changes in pmr intensities characteristic of the native state that were observed in the initial pmr study of the thermal denaturation by McDonald and Phillips.[9] Bradbury and King[14] examined pmr spectra of lysozyme solutions at pH 2.8 as a function of urea concentration and concluded that the unfolding of the protein proceeds in at least two steps. In this paper we explore in some detail the pmr spectral changes that accompany lysozyme denaturation and renaturation and examine the extent to which paths of unfolding and folding of the protein can be deduced from them.

## Experimental Section

Pmr spectra were obtained with a Varian Associates high-resolution spectrometer which operates at a frequency of 220 MHz. The temperature in the sample zone is established by a stream of nitrogen of controlled but variable temperature and was regulated within ±0.5° in our experiments. Temperatures were measured from the chemical shifts of the resonances of ethylene glycol that were examined before and after the acquisition of each protein spectrum. Internal referencing with small amounts of tetramethylammonium ion (TMA) added as the chloride was employed. However, in the spectra presented, the zero chemical-shift position is that of the more standard reference, sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). The latter reference obscures an important region of the pmr spectrum of lysozyme and was used only to establish that the methyl resonance of internal TMA in a 10% solution of lysozyme is at 3.23 on the scale referred to DSS. Positive chemical shifts are to low field from the reference position. Experiments with and without TMA indicated that the small amount of reference material in the protein solution had no influence on the behavior of lysozyme as manifested by the protein pmr spectrum. Signal-to-noise characteristics of many of the spectra were improved by employing a computer of average transients (Varian Associates C-1024). The lysozyme used in these studies was purchased from Worthington Biochemical Corporation.

## Results

Characteristics of pmr spectra of proteins appear to have great potential for elucidating details of denaturation and folding of these molecules. Since a proton resonance position in a pmr spectrum is readily influenced by the nature and arrangement of the bonded and nonbonded atoms in the immediate environment of the hydrogen atom, each resonance of the pmr spectrum of a molecule serves as a probe of the chemical structure of a region of the molecule. To derive such structural information it is essential that individual resonances be resolved, that these resonances are assignable to specific hydrogen atoms in the molecule, and that the chemical shifts of the resonances can be rationalized in terms of molecular structure. These criteria hold for proteins just as for small molecules, but are more difficult to achieve for the former because of the great number of proton resonances (mostly overlapping) that are exhibited by the many hydrogen atoms of these complex molecules. Nevertheless, with pmr spectrometers now available, resonances of individual or equivalent groups of protons of proteins often can be resolved. The rather subtle environmental pertubations brought about by the folding of proteins frequently are reflected in chemical-shift displacements. To the extent that such resolved resonances can be identified with specific protons of particular amino acid residues and to the extent that these proton probes are distributed widely throughout the three-dimensional structure of the protein, inferences can be drawn on the local disordering caused by a denaturation variable (temperature, pH, or denaturant concentration) and on the simultaneity of unfolding of various regions of the protein. In this section, changes in the pmr spectrum of HEW lysozyme that accompany denaturation are examined. In the next, these results are discussed in terms of denaturation mechanisms.

**Thermal Duration in $D_2O$, pD 5.0.** Regions of the pmr spectrum of HEW lysozyme are shown in Figure 1 for temperatures from 35 to 80°. The protein was heated at 80° for 15 min before pmr examination to replace, by deuterons, N*H* protons which exhibit resonances from 6 to 11 ppm in the spectrum of the native protein. While most of the N*H* protons exchange during sample preparation, some persist for days if the sample temperature is kept below 45°. The region of resonance absorption from 3 to 6 ppm was not studied since absorption in this region, arising mainly from $\alpha$-C*H* protons, is seriously obscured by the residual *H*DO solvent resonance. The spectra shown, therefore, comprise C*H* resonances of the residue side chains of lysozyme. The spectral features are essentially invariant with temperature from 35 to 65° and are characteristic of the native state of the protein. At the low temperatures, resonances of lysozyme are slightly broadened and less well resolved because of aggregation of native lysozyme that is favored by low temperatures and pH values above about 5.[15] The aggregated forms, possessing greater rotational correlation times, are expected to exhibit broader resonances than the monomer. The observed pmr spectrum appears to be an "exchange averaged" spectrum (see below) of the monomeric and aggregated forms (*i.e.*, aggregation and disaggregation are rapid processes on the nmr time scale). Actually, this "aggregation broadening" is minor at pD 5 at temperatures above 35° and decreases at lower pD values; it becomes quite prominent at pD values above about 5.5.

Except for the conditions of low temperature and high pD noted above, lysozyme appears to exist in aqueous solution in an unaggregated monomeric form. No changes in resonance widths or in other spectral characteristics were observed when a 1% lysozyme solution was examined and the widths of resolved resonances of the native protein are typical of those commonly observed for a diamagnetic globular protein with a molecular weight around 15,000.

As the temperature is increased from 65 to 80°, the pmr spectrum of lysozyme changes markedly as

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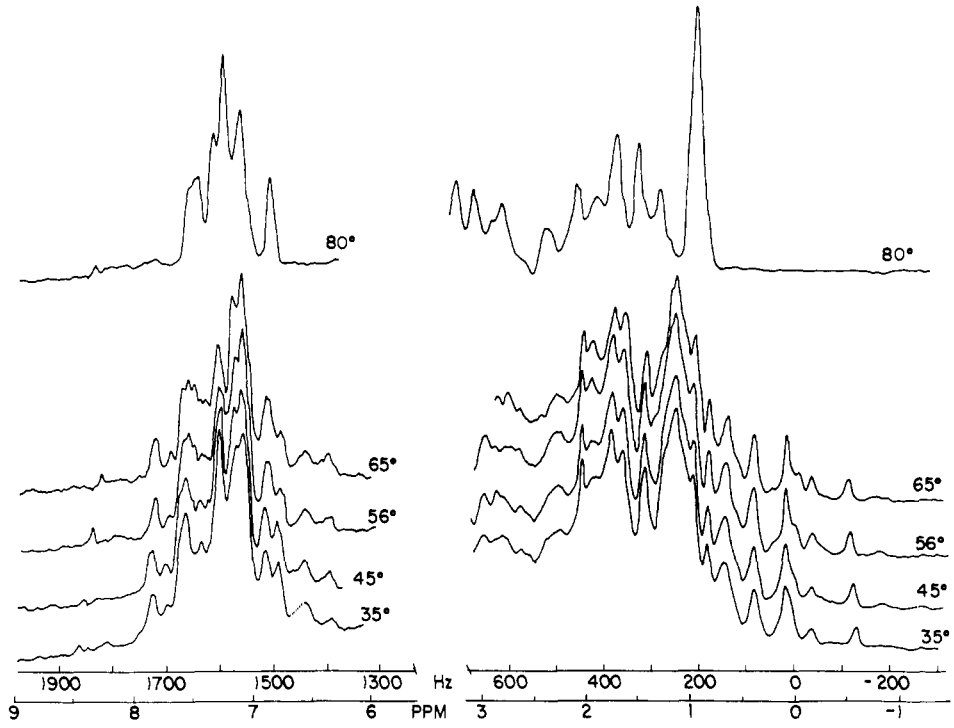


Figure 1. Pmr spectra of lysozyme (10% in $D_2O$, pD 5.0). N*H* protons preexchanged. Low-field amplification greater than high field.

the protein denatures. At 80° and beyond, the spectrum is again invariant, indicating that the protein is entirely in the thermally denatured form and that the spectrum at 80° is characteristic of this unfolded state. Precisely what this state is, however, requires further consideration.

Analysis of the pmr spectrum of thermally denatured HEW lysozyme has been given elsewhere.[16] The analysis was based on the finding that the observed spectrum could be accurately reconstructured by assuming that all protons of a given chemical type in the protein exhibit resonance at the same field position and at a field position similar to that of the same proton type in the pmr spectrum of the appropriate monomeric amino acid in neutral $D_2O$. For example, all 36 alanine methyl proton resonances of thermally denatured lysozyme appear to occur at 1.41 ppm regardless of the positions of the 12 alanine residues in the primary sequence. For this to be the case, the alanyl methyl side chains in the polypeptide must experience similar environments (as regards factors that affect pmr chemical shifts) and further, environments that are similar to that of alanine in neutral $D_2O$. We conclude, therefore, that in the thermally denatured state the side chains of the amino acid residues of lysozyme are in an environment that is defined by the solvent and that the resonance positions are relatively unaffected by neighbor residues. This form does not appear to retain elements of folded structure that influence resonance positions of side-chain protons.

Returning to the spectrum at 80° in Figure 1, the strong resonance with maximum intensity at 197 Hz arises from methyl protons of leucine, isoleucine, and valine residues, the resonance at 270 Hz from methyl protons of threonine, the resonance at 310 Hz mainly from methyl protons of alanine, and the resonance at 365 Hz from protons of lysine, arginine, and leucine residues. The methyl proton resonances of methionine residues occur at 454 Hz. The resonances from 1500 to 1700 Hz arise from aromatic C*H* protons of tryptophan, tyrosine, phenylalanine, and histidine. The weak resonance at about 1835 Hz is assigned to the imidazole C-2 proton of the single histidine residue of lysozyme. The position of this resonance depends on the state of protonation of the imidazole ring and shifts from about 1740 to 1890 Hz as pD is decreased from 7 to 3. Further, the C-2 histidine proton exchanges slowly in $D_2O$ so that the intensity of this resonance is reduced significantly in the time (about 90 min) required to obtain a spectrum such as the low-field spectrum at 80° in Figure 1. The extent of this exchange depends also, of course, on the time–temperature history of a sample between its preparation in $D_2O$ and examination.

The pmr spectra of the folded, biologically active form of lysozyme are very different from and much more complex than the spectrum of the thermally denatured form (compare the 35–65° spectra of Figure 1 with the 80° spectrum). In the native state, many of the residue side chains are in environments defined by surrounding elements of the folded protein rather than by the solvent. Resonances of the protons of these side chains are likely to be shifted from the "denatured" positions to varying extents, depending on the detailed nature of the environments. Several completely or partially resolved resonances are observed in the field region from 100 to −200 Hz. These are attributed mainly to methyl protons that in the native protein lie close to the faces of aromatic rings so that their resonance positions are shifted to high field by the ring-current fields associated with the aromatic structures.

As reported in earlier studies[10,11] assignment of these resonances to specific methyl groups is not

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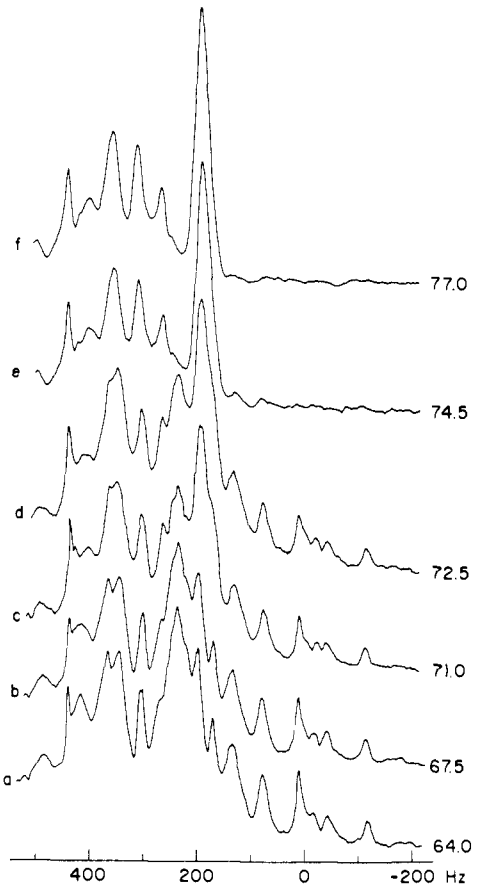


Figure 2. High-field pmr spectra of lysozyme (10% in $D_2O$, pD 5.0) through thermal denaturation. Amplification at 74.5 and 77.0° about one-half amplification at lower temperatures.

straightforward. However, tentative assignments of some of them have been made by combining information from several sources. The structure of lysozyme deduced from X-ray spectroscopy[1] was examined to determine which methyl groups are close to aromatic ring faces, and the approximate "ring-current" shifts expected for the protons of these methyl groups were estimated using for each aromatic ring the "ring-current" field calculated by Johnson and Bovey[17a] for benzene.[17b] These estimations predict qualitatively which methyl resonances should be shifted to high field but are not sufficiently quantitative for unique assignments. Next, examination of pmr spectra of solutions of lysozyme containing $Co^{2+}$ showed that the spectra were modified in a regular manner as the $Co^{2+}$ concentration was increased.[18] Some resonances were found to shift to high field, others to low field. These studies helped greatly in dissecting out the component resonances in the partially resolved field region from −10 to 50 Hz. The $Co^{2+}$-induced shifts appear to be caused by pseudocontact dipolar interactions between protein protons and paramagnetic $Co^{2+}$ bound at the glutamic acid-35 residue (the active site). Consequently, the direction and magnitude of a $Co^{2+}$-induced shift depends on the distance between a proton and the bound $Co^{2+}$ and on the angle between this distance vector and the ligand field axis of the bound ion. Directions and magnitudes of the $Co^{2+}$-induced shifts of the methyl resonances in the high-field region provided additional information for assigning these resonances. For example, resonances having about the same $Co^{2+}$-induced shifts could arise from protons in the same region of the protein, perhaps from the same residue, but this could not be the case if the magnitudes of the shifts were considerably different or were in opposite directions. Further information was obtained from resonance widths. Where the half-width of a single resonance (no unresolved components) is 6–10 Hz, as is the case for native lysozyme resonances, resolved methyl resonances of methionine can be identified by the fact that they are sharper than those of other methyl resonances because they are not broadened by unresolved fine structure arising from proton spin-coupling interactions. Lastly, small shifts of two of the high-field methyl resonances of lysozyme with temperature suggest that these resonances may be associated with the same residue.

From consideration of the combined information from these several types of observations, we have made the following methyl proton resonance assignments: resonances at −124 and −8 Hz to leu-17, at −38 and 0 Hz to ile-98, and two resonances at about 15 Hz to met-105 and one methyl group of leu-8. Regardless of the detailed correctness of these assignments, clearly these high-field methyl resonances arise from groups widely distributed in the protein molecule. These resonances provide very sensitive probes of conformation changes since their resonance positions are determined by ring-current fields of nearby residues. These fields are sensitive functions of distance and angle.[17] Changes of only a few hundredths of an ångström of the relative positions of the methyl groups assigned above with respect to their neighboring aromatic residues should produce readily observable shifts of the associated resonance. For example, the methyl group of met-105 appears to be affected by the ring-current fields of trp-28, trp-108, trp-111, and tyr-23. Changes in position of any of these residues in the native protein structure would cause the sharp methionine methyl resonance at 15 Hz to shift. Leu-17 methyl groups are mainly influenced by trp-28 with a minor perturbation by tyr-20. The latter residue lies on the surface of lysozyme and the small temperature-induced shifts of the leu-17 methyl proton resonances may result from a change in the orientation of the tyr-20 aromatic ring as temperature is increased.

Changes in the high-field region of the pmr spectrum of lysozyme as the solution was heated from 64 to 77° are shown in Figure 2. This solution was not heated after preparation until it was raised to 64° to begin the pmr examination. As temperature was increased, the spectrum characteristic of the native state decreased in intensity while the spectrum characteristic of the thermally denatured state observed at temperatures >80° grew in intensity. At temperatures through the thermal denaturation transition, each observed spectrum appears to be a composite of two spectra, one deriving from completely thermally denatured protein and one from native protein, so that the form of the spectrum depends only on the relative amounts of the two states of the protein that are present at a given temperature.

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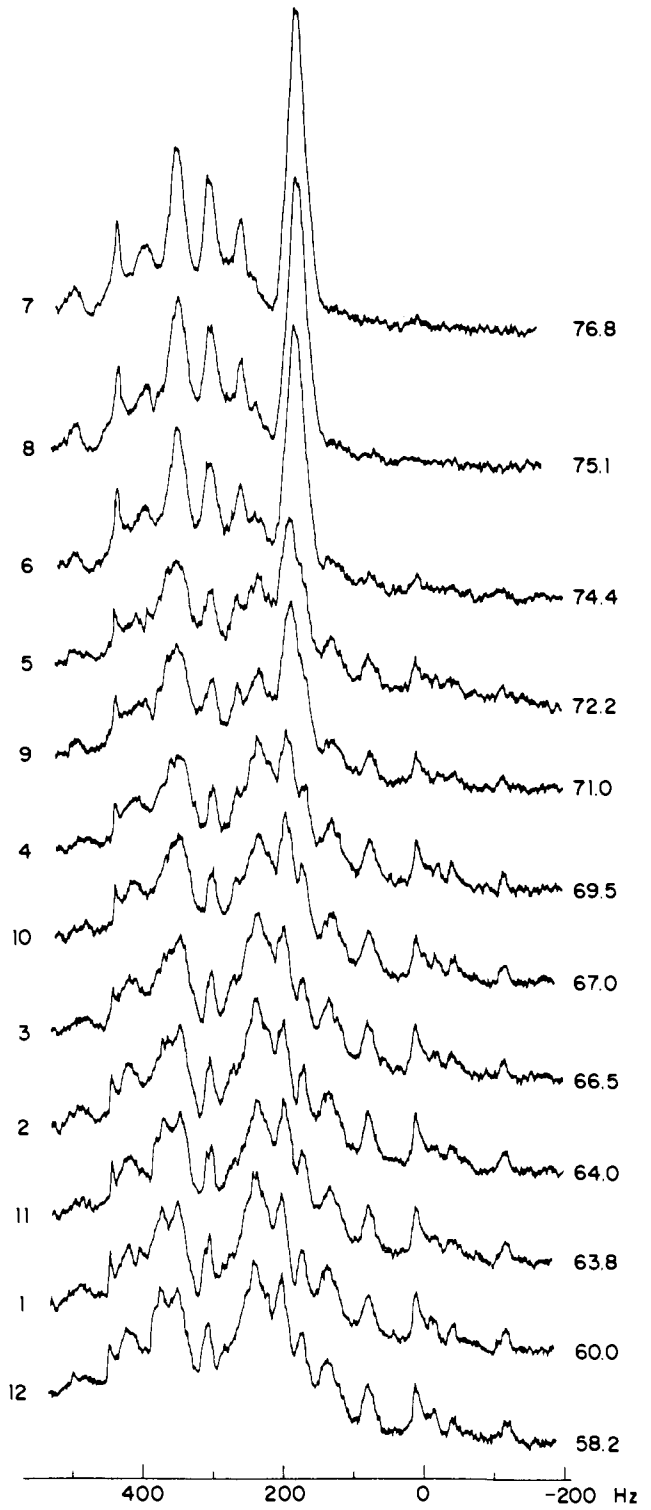



Figure 3. High-field single-scan pmr spectra of lysozyme through denaturation and renaturation (10% in $D_2O$, pD 5.0). Numbers on left refer to order of acquisition, on the right to the temperature (°C).

A major question to be answered at this point is whether denaturation is completely reversible for the conditions of our experiments. The lysozyme sample that provided the pmr spectra of Figure 2 was examined further by obtaining the pmr of the same field region as the sample was cooled in several steps from 77 to 65°. For the most part, the thermal-denaturation process appears to be completely reversible. At each temperature through renaturation the pmr spectrum is a composite of the spectra characteristic of the thermally denatured and native states, with the proportion of the latter increasing regularly as the sample is cooled from 77 to 65°. However, at each temperature the ratio of the denatured to native states was slightly higher than during the heating experiment portrayed in Figure 2. At 65° and lower temperatures, the pmr spectra indicated that about 5% of the lysozyme persisted in the denaturated state, but the remainder of the protein was renatured to a native form that exhibits a pmr spectrum identical with that observed for the native protein before thermal denaturation. Further investigation showed that the amount of lysozyme that is not rapidly renatured on cooling from the thermally denatured state is dependent on the length of time that the protein is at high temperature in the denatured state. Each spectrum in Figure 2 represents an accumulation of many individual spectral traces by a computer of average transients to obtain spectra with a satisfactory signal–noise characteristic. Consequently, it was necessary to hold the lysozyme solution at each temperature approximately 1 hr so that in the course of the denaturation and renaturation experiments the protein was exposed to high temperature in the denatured state for several hours. Thus, the buildup of protein that is not rapidly renaturable is a rather slow process.

As an additional test of the reversibility of thermal denaturation of lysozyme, a fresh sample of lysozyme (10% in $D_2O$, pD 5.0) was prepared and, without any prior heating, pmr spectra of the high-field region were obtained at several temperatures as the protein was heated from 60 to 77° and then was cooled back down to 58°. In this experiment, however, only a single spectral trace was taken at each temperature and at the conclusion of each trace, the sample was held at room temperature until the spectrometer temperature had been readjusted for the next temperature. In this way the time required for thermal equilibration of the sample in the spectrometer and examination of the pmr spectrum was reduced to 12–15 min at each temperature. These spectra are shown in Figure 3 where the numbering refers to the order in which the spectra were acquired. In this experiment it appears that thermal denaturation is completely reversible. The final spectrum at 58° appears identical in intensity and spectral characteristics with the initial spectrum at 60°. In the temperature range from 65 to 80°, the apparent ratio of denatured to native protein increases regularly with increasing temperature (taking into account that the temperature measurements have an estimated error of ±0.5°), regardless of whether the traces were obtained as the protein was denatured or renatured. Equilibrium between denatured and native protein is established in less than 12 min at temperatures above 65° since in the experiment just described if a second trace was obtained at a given temperature, it was identical with the first. Additional evidence for the rapid conversion between the denatured state and native state is provided by the fact that if the protein is cooled from 80 to 4° and examined within 12 min, the pmr spectrum reflects complete renaturation to the native state.

To verify further the complete reversibility of the thermal denaturation of lysozyme with pmr spectra

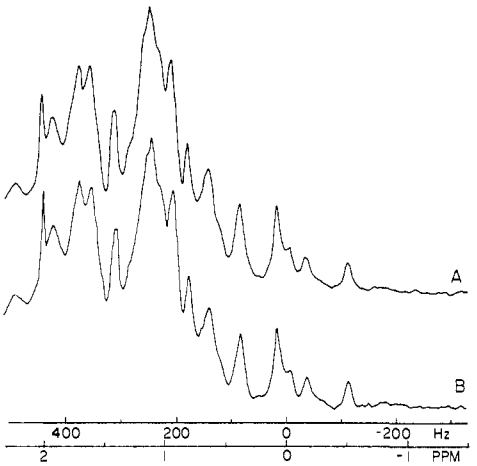



Figure 4. High-field pmr spectra of lysozyme at 61°: A, before denaturation; B, after renaturation (10% in $D_2O$, pD 5.0).

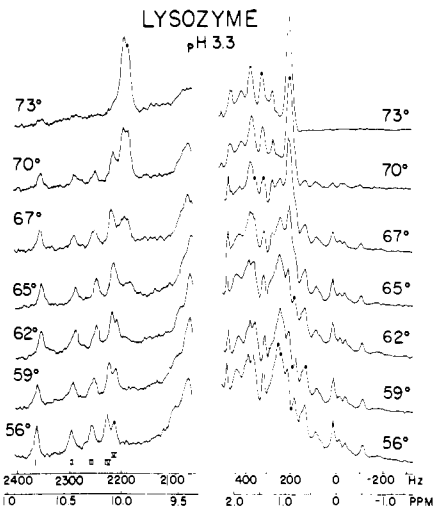



Figure 5. Pmr spectra of lysozyme through thermal denaturation (10% in $H_2O$, pH 3.3). Low-field amplification much greater than high field.

with better signal–noise characteristics than those of Figure 3, the following experiment was done. A solution of lysozyme (10% in $D_2O$, pD 5.0), freshly prepared without any heat treatment, was examined at 61° using computer averaging to obtain pmr spectrum A in Figure 4. The spectrometer temperature was raised to 77° and a single pmr trace was obtained to confirm that the protein was converted to the thermally denatured state. The spectrometer temperature was then readjusted to 61° and spectrum B of Figure 4 was obtained. Spectra A and B appear to be identical in intensity and both exhibit the spectral characteristics that are observed when the protein is entirely in the native state. Hence, we conclude that if prolonged heating of lysozyme in the denatured state is avoided, the process of thermal denaturation is completely reversible for our experimental conditions.

To summarize the observations on lysozyme at pD 5.0, it appears that through the processes of thermal denaturation or renaturation two forms, distinguishable by pmr spectroscopy, exist in equilibrium which is established in less than 12 min. On the other hand, since discrete pmr spectra displaying no evidence of exchange averaging or broadening are observed for the native and denatured protein when they are present together during denaturation or renaturation, a minimum value can be assessed for the lifetime of a particular molecule in either state.[19] In the transition region, pairs of resonances separated by about 200 Hz have been identified with unique methyl groups distributed between native and denatured forms of the protein. Therefore, the rate of interconversion of these forms is less than $1 \times 10^3$ $sec^{-1}$ or, in other words, the lifetime of a molecule in either state exceeds $8 \times 10^{-4}$ sec. Other "native" and denatured" resonances are discussed below that have been assigned to particular protons of histidine and tryptophan residues. Again, the "native" and denatured" resonances for the same proton appear as discrete, resolved resonances when both the denatured and native forms are present. For two indole N*H* resonances of tryptophan of lysozyme in the thermal denaturation range, one resonance characteristic of the native and the other of the denatured form, the separation amounts to only 30 Hz (Figure 5). Resolution of these two peaks indicates that the rate of interconversion between the two forms under these conditions must be less than $2 \times 10^2$ $sec^{-1}$.

**Thermal Deuteration in $H_2O$, pH 3.3, and $D_2O$, pD 3.3.** Thermal denaturation of lysozyme is manifested strikingly in resolved and assignable resonances of the indole N*H* protons of the six component tryptophan residues. To observe resonances of such exchangeable protons, slightly acidic $H_2O$ must be employed as solvent.[13] Pmr spectra of the high-field and tryptophan N*H* field regions obtained for lysozyme at temperatures from 56 to 73° are shown in Figure 5. The spectra were acquired in order of increasing temperature on lysozyme solutions which were not previously heated. At each temperature, the high-field spectrum was recorded before and after acquisition of the low-field spectrum to confirm that no time-dependent change had occurred. To minimize the effects of time-dependent irreversible denaturation discussed above, one lysozme sample was used for experiments at 56, 59, and 62°, and a second sample for temperatures from 65 to 73°.

The 73° spectrum of Figure 5 is characteristic of the thermally denatured state. The N*H* protons of the six tryptophan residues all exhibit resonance at 2190 Hz, irrespective of position in the primary sequence. Upon renaturation, the 2190-Hz resonance splits into six of unit intensity, reflecting the nonequivalent environments of the tryptophan residues in the folded, native protein. Only five of the six indole N*H* protons of lysozyme have been identified in the spectrum of lysozyme (I–V in the 56° spectrum of Figure 5). The sixth resonance presumably occurs in the complex amide N*H* region of resonance absorption. These resonances have been assigned to specific tryptophan

(19) The relationship between exchange rates and nmr observables is important to this discussion and, consequently, is briefly illustrated here. If a proton by some exchange process spends its time equally in two environments a and b characterized by chemical shifts in hertz of $\delta_a$ and $\delta_b$, then two resonances of equal intensity are observed for the case of "slow" exchange when the rate of environmental exchange is less than $2\pi(\delta_a - \delta_b)$. Only a single resonance located midway between $\delta_a$ and $\delta_b$ is observed for the "fast" exchange situation, *i.e.*, rate of exchange exceeds $2\pi(\delta_a - \delta_b)$. "Exchange-broadened" versions of the two resonances or the single resonance are observed when the exchange rate is comparable to $2\pi(\delta_a - \delta_b)$.

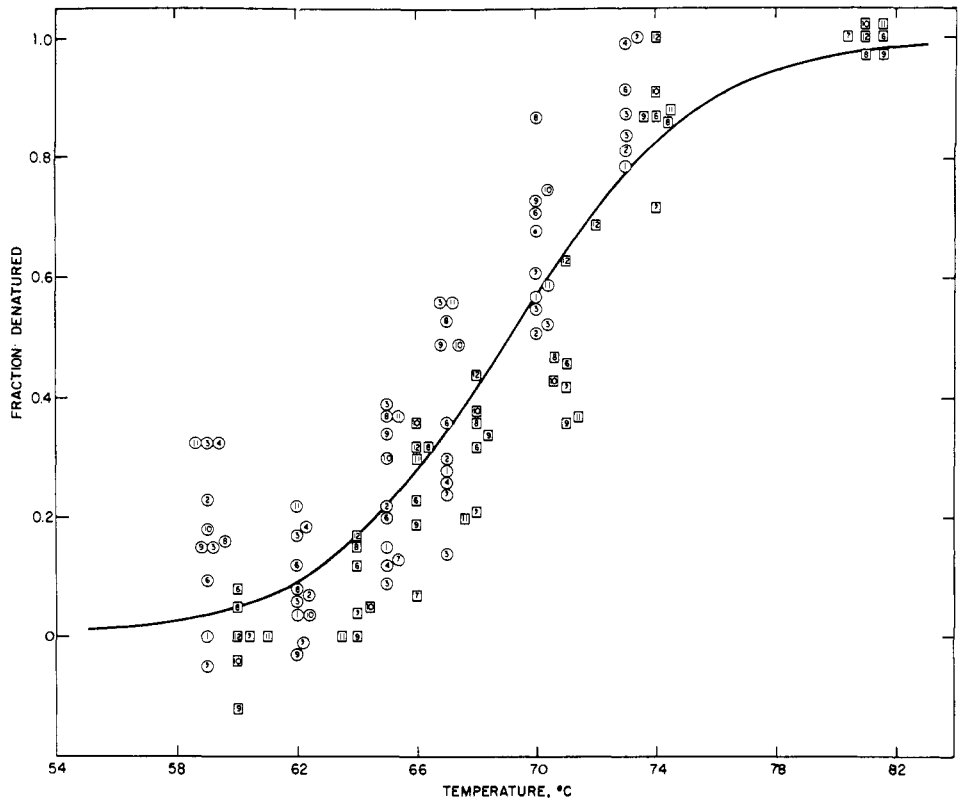



Figure 6. Fraction of lysozyme denatured as a function of temperature from resonance intensities. Circles are from observations in $H_2O$, pH 3.3; squares are from observations in $D_2O$, pD 3.3: (1) tryptophan N*H* "denatured"; (2) tryptophan native N*H* I; (3) tryptophan native N*H* II; (4) tryptophan native N*H* III; (5) tryptophan native N*H* IV and V; (6) peak height at 240 Hz, $H_{240}$; (7) peak height at 200 Hz, $H_{200}$; (8) region A; (9) region B; (10) region C; (11) region D; (12) histidine-15, C-2.

residues from differential deuterium exchange rates, inhibitor perturbations, and chemical modification studies.[13] Specifically, these assignments are: I (trp-28 or trp-111), II (trp-108), III (trp-62), IV (trp-123), and V (trp-63). Thermal denaturation of lysozyme occurs from 60 to 73° at pH 3.3. It is apparent that the tryptophan N*H* pmr spectra in this temperature range are superpositions of spectra characteristic of the native and denatured states and that the extent of thermal denaturation at a particular temperature indicated by the high-field and low-field spectral regions are very similar, if not identical.

To pursue this point, pmr intensities (absorption integrals) of the tryptophan N*H* resonances and certain of the high-field resonances of the spectra of Figure 5 have been used to calculate the fraction of lysozyme denatured as a function of temperature. The results of these calculations are plotted in Figure 6. The resonance intensity $R_0$ of a single tryptophan indole N*H* proton of the native protein was obtained by averaging the intensities of resonances I, II, III, IV, and V (Figure 5) at 56°. If the intensity of resonance I at a higher temperature was $R_I$, the fraction of the protein denatured was taken as $1 - (R_I/R_0)$. The same procedure was used for resonance II and III. The combined intensities of resonances IV and V were measured and the fraction denatured was calculated as $1 - (R_{IV,V}/2R_0)$. The intensity of the "denatured" tryptophan N*H* resonance, $R_D$, was also measured; the fraction denatured here was taken as $R_D/6R_0$. In the high-field region, a qualitative indication of denaturation is provided by the decrease of resonance peak height, $H_{240}$, at 240 Hz, or by the increase of the resonance peak height, $H_{200}$, at 200 Hz as temperature is increased. The change in $H_{200}$ with temperature has been used by others to follow protein denaturation by pmr.[20]

The resonances at both 200 and 240 Hz are envelopes of many unresolved resonances and, therefore, reflect some sort of average behavior of the protein molecule rather than conformational change in a specific region. Since they are envelopes of resonances, they must be interpreted with caution; the peak height values may depend on small changes of component resonance positions and resonance widths as well as resonance intensities. While $H_{240}$ appears to arise almost entirely from "native" resonances, both "native" and "denatured" resonances occur at the $H_{200}$ position so that as the result of denaturation the intensities of the native components are diminished and the intensities of the denatured components are increased (the peak position shifts from 205 Hz). Nonetheless, for lysozyme, changes of $H_{240}$ and $H_{200}$ appear to correspond quite closely to the process of denaturation as measured by individual resolved resonances and they have been included as indicators of denaturation in the data shown in Figure 6. For $H_{240}$, the fraction of denatured protein was taken as $1 - (H_{240}/H_{240}^*)$ where $H_{240}^*$ was the observed value at 56°. For $H_{200}$, the fraction denatured was calculated from $(H_{200} - H_{200}^{\pm})/(H_{200}^* - H_{200}^{\pm})$ where $H_{200}^*$ and $H_{200}^{\pm}$ are the observed values at 73 and 56°, respectively. In addition, extents of denaturation were estimated from intensity changes of four regions (A, B, C, and D) of the high-field spectra

(20) For example, see D. P. Hollis, G. McDonald, and R. L. Biltonen, *Proc. Nat. Acad. Sci. U. S.*, **58**, 758 (1967).

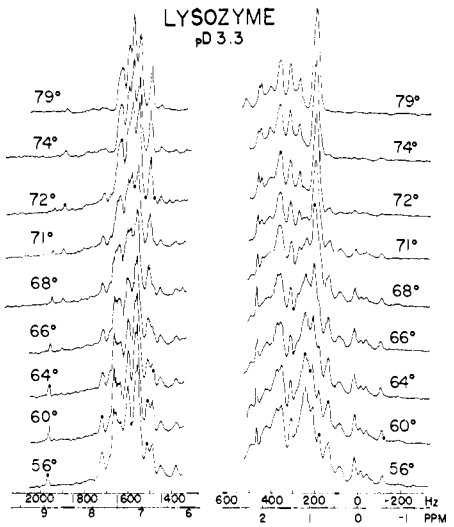



Figure 7. Pmr spectra of lysozyme through thermal denaturation (10% in $D_2O$, pD 3.3).

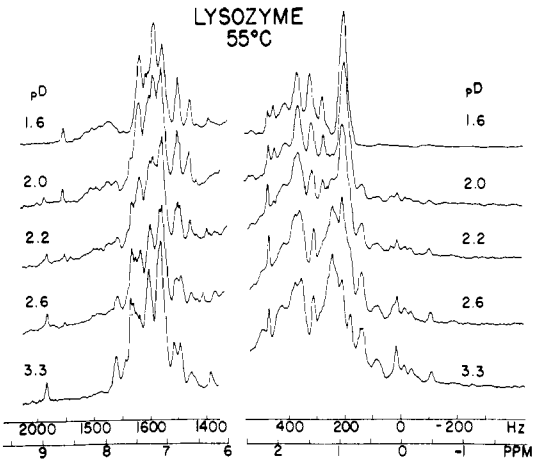



Figure 8. Pmr spectra of lysozyme through acid denaturation (10% in $D_2O$, 55°).

that arise entirely from "native" resonances. Region A (104–163 Hz) comprises about 23 proton resonances, including several methyl resonances (possibly leu-8, leu-56, and ile-55). Region B (57–104 Hz) contains about ten proton resonances which probably include methyl group resonances of thr-51 and ile-88. Region C (−90 to 57 Hz) includes about 18 protons, 15 of which have been assigned to methyl groups of met-105, ile-98, leu-8, and leu-17. Region D contains only the methyl resonance of leu-17. For each region the intensity for the completely native state, $R_N$, was obtained from the observed intensity at 56°. At higher temperatures, the fraction denatured was obtained from $1 - (R/R_N)$, where $R$ is the observed integral intensity at a particular temperature. The solid line in Figure 6 represents the calculated relationship between fraction denatured and temperature for the process N $\rightleftarrows$ D for $\Delta H = 73.5$ cal/mol and the midpoint of the denaturation where the concentrations of N and D are equal is at 69.0° (see Discussion).

A parallel study of thermal denaturation of HEW lysozyme in $D_2O$ at pD 3.3 was made in which pmr spectra of the high-field and aromatic C*H* regions were acquired at temperatures from 56 to 79° (Figure 7). A single solution of lysozyme (10%) was used that was heated briefly at 80° prior to pmr examination so that N*H* resonances of unexchanged N*H* protons would not obscure the aromatic C*H* spectrum. Fresh aliquots of this solution were used for each temperature to minimize the loss of intensity of the his-15 C-2 resonance by exchange. Reversible thermal denaturation from 60 to 75° is accompanied by changes in the high-field spectrum similar to those described at pD 5.0. The low-field region exhibits parallel changes that are most clearly seen for the his-15 C-2 resonance. For the native protein the resonance occurs at 1940–1964 Hz, for the denatured protein at 1895–1905 Hz. Through thermal denaturation or renaturation both C-2 resonances are observed; their relative intensities reflect the ratio of the native and denatured states that are present in equilibrium at a particular temperature. It appears that his-15 and the high-field hydrophobic residues reflect the same structural changes at the same temperatures. It can be seen in Figure 7 that a number of changes occur in the 1400–1800-Hz aromatic region of resonance absorption of lysozyme as a result of thermal denaturation. This region of resonance absorption in both native and denatured forms is too poorly resolved to isolate useful conformational markers.

Again, the fraction of the lysozyme denatured at temperatures from 60 to 75° was estimated from intensity changes of various resonances and resonance regions of the pmr spectra of Figure 7. The results of these analyses are also shown in Figure 6. In the case of the histidine C-2 resonances the fraction denatured was taken as $R_D/(R_D + R_N)$ where $R_D$ and $R_N$ are the integral intensities of the "denatured" and "native" resonances, respectively. The computations for the high-field spectra were analogous to those described earlier.

The detection sensitivity of current pmr spectrometers even when enhanced by extensive computer averaging is insufficient to provide highly reproducible intensity data for single protein resonances. A variation of 20% or so in the integrated intensity of a resonance examined repeatedly with the same nominal experimental conditions is not uncommon. Further, the uncertainty in the sample temperature in our experiments was ±0.5°. Consequently, the data presented in Figure 6 show considerable scatter. Nonetheless, some conclusions can be drawn. Within the accuracy of the method, extent of denaturation exhibits the same dependence on temperature in $D_2O$ at pD 3.3 as in $H_2O$ at pH 3.3. Intensity changes are plotted for resonances that are sampling conformational changes in many regions of the protein molecule. At a particular temperature the degree of denaturation indicated by the various resonances varies up to 40%. However, examination of the separate data for an individual conformational probe through denaturation indicates that there is a random scatter of points around the solid line. Thus, we conclude that the variation in data points at a particular temperature is measurement error. There is no evidence that there are different denaturation–temperature relationships for different parts of the protein molecule.

**Acid and Guanidine Denaturation.** Lysozyme denatures at lower temperatures as the protein solution is

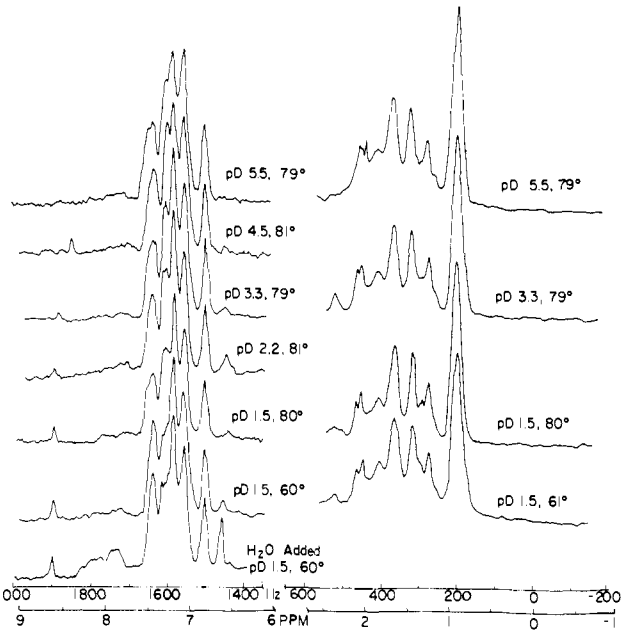



Figure 9. Pmr spectra of denatured lysozyme with variation of temperature, pD, and $H_2O$ concentration.

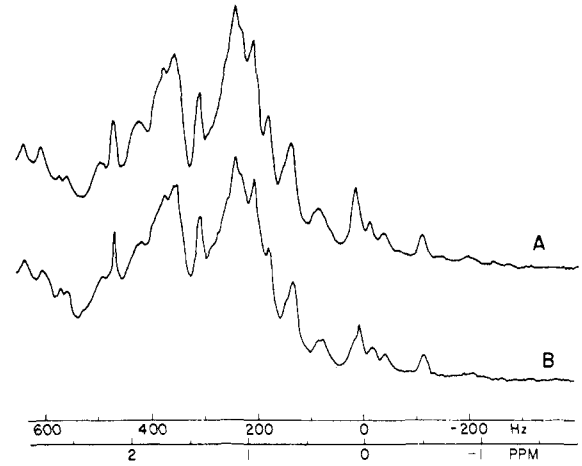



Figure 10. Pmr spectra of lysozyme at pD 3.3: A, before acid denaturation; B, after renaturation (10% in $D_2O$, 55°).

made more acidic and, in fact, pH can be employed as the denaturation variable at constant temperature.[3] Pmr spectra of lysozmye in $D_2O$ at 55° were acquired through acid denaturation as pD was reduced from 3.3 to 1.6 (Figure 8). The spectra at pD 3.3 and 1.6 are characteristic of the native and denatured states, respectively. The changes in the spectra for intermediate pD values parallel those observed during thermal denaturation; that is, they are weighted superpositions of the "native" and "denatured" spectra.

The aromatic region of the pmr spectrum of the denatured protein at pD 1.6 (55°) exhibits a strong resonance at 1460 Hz and a group of unresolved resonances from 1700 to 1850 Hz that were not noted in previous pmr spectra of the denatured form. Since these resonances might reflect elements of folded structure of the protein in the denatured state that are not retained at higher pD or temperature, additional spectra of the denatured form were obtained for other values of these variables (Figure 9). In these experiments, the new resonances were observable (but weak) at pD values below about 5 at 80°. They became somewhat sharper as pD or temperature were decreased but did not change appreciably in intensity. Their intensities were found, however, to be proportional to the amount of $H_2O$ in the $D_2O$ solvent. We conclude that these resonances do not reflect differences in the structure of lysozyme denatured under different conditions but arise from N*H* protons. Based on studies of amino acids in $H_2O$, the resonance at 1460 Hz is assigned to side-chain N*H* protons of the 11 arginine residues, and the resonances from 1700 to 1850 to amide N*H* protons. These protons exchange readily with the $D_2O$ solvent so their intensities depend on the "proton impurity" content ($H_2O$ and HDO) of the solvent. In acidic solutions, however, their exchange rates with water are sufficiently slow so that their resonances are not "exchange averaged" with the solvent proton resonance. As pD is increased, "exchange broadening" occurs and at about pD 5 (80°) the exchange rate becomes so fast that the arginine and amide N*H* resonances are no longer detectable.

The reversibility of the acid denaturation of lysozyme was examined as follows. The high-field pmr spectrum of a solution of lysozyme (10% in $D_2O$) was obtained at pD 3.3 (55°) and is shown as spectrum A of Figure 10. The pD of the solution was reduced to 1.5 and a single scan spectrum was acquired at 55° to demonstrate that the protein was converted to the denatured state. The pD was readjusted to pD 3.3 and spectrum B of Figure 10 was obtained employing the same conditions as for spectrum A. Spectra A and B appear to be substantially identical and indicate that the pD denaturation as performed in this experiment is fully reversible.

Finally, the denaturation of lysozyme induced by adding guanidine hydrochloride to the protein solution was studied. High-field pmr spectra of lysozyme (10% in $D_2O$, pD 5.5, 40°) in solutions containing guanidine at various concentrations up to 6 *M* are shown in Figure 11. Denaturation becomes appreciable at guanidine concentrations above 3 *M* and appears to be essentially complete in 6 *M* guanidine. The spectra acquired at guanidine concentrations between 3 and 6 *M* appear again as composites of the "native" and "denatured" lysozyme spectra. However, there were some changes in the pmr spectrum of lysozyme observable after the first addition of guanidine (1 *M*) as may be seen by comparing spectra 9 and 10 of Figure 11. It is not clear whether these changes are the consequence of a conformation change or result merely from the change of the solvent environment about residue side chains that are on the exterior of the protein.

## Discussion

The pmr spectrum of denatured lysozyme indicates that the denatured protein is extended in solution with all of the residue side chains in solvent environments that are equivalent (so far as effects on pmr chemical shifts are concerned) so that resonances of all protein hydrogen atoms of a given chemical type are equivalent. The pmr spectrum of folded, native lysozyme is quite different from that of the denatured form because of substitution in the former of closely juxtaposed side chains for solvent as the dominating environmental influence. Changes in chemical shifts

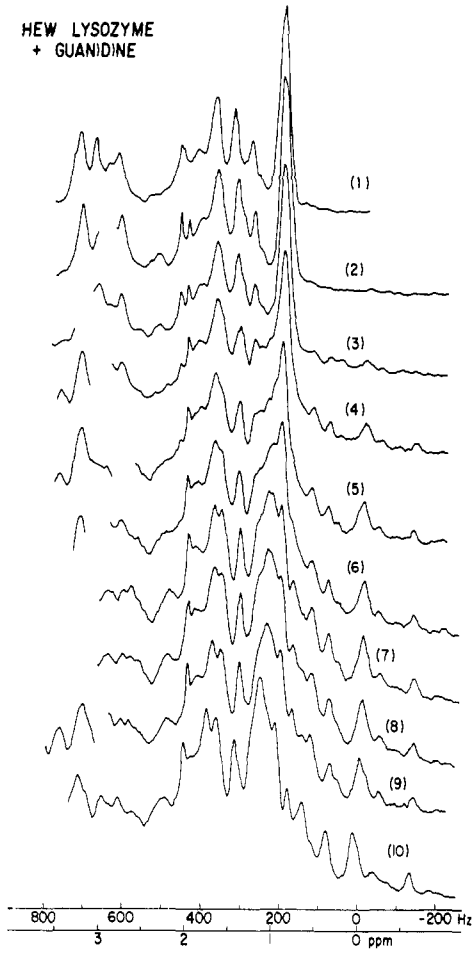



Figure 11. High-field spectra of lysozyme through guanidine hydrochloride denaturation (10% in $D_2O$). Spectrum (1) pD 5.9, 79°; all others pD 5.5, 40°. Guanidine concentrations: (1) 0, (2) 6 *M*, (3) 5 *M*, (4) 4.5 *M*, (5) 4.25 *M*, (6) 4 *M*, (7) 3 *M*, (8) 2 *M*, (9) 1 *M*, (10) 0. Amplitudes not constant between spectra.

of individual protons are introduced by short-range intramolecular shielding perturbants such as anisotropy fields and hydrogen bonding. A number of resolved resonances in the pmr spectrum of native lysozyme have been assigned to specific amino acid residues of the polypeptide chain. These protons are distributed widely through the three-dimensional structure of the protein. Individually they are sensitive to changes in the local protein environment; in aggregate they provide a sensitive index of changes in the three-dimensional conformation of the entire molecule.

In thermal, acid, or guanidine denaturation the pmr spectra of lysozyme through the transition range of the denaturant parameter can be represented as weighted superpositions of spectra characteristic of the native conformation, N, and a denatured form, D. Interconversion between the two forms is reversible (if prolonged exposure to denaturing conditions is avoided) in accordance with the equilibrium N $\rightleftarrows$ D. Equilibrium is established in less than 12 min (at pD 5.0, 60–70°). No pmr resonances attributable to intermediate states were observed in the transition regions. At pH (or pD) 3.3 intensity changes of individual "native" and "denatured" resonances from 56 to 81° indicated that at a particular temperature the degree of unfolding is the same for all parts of

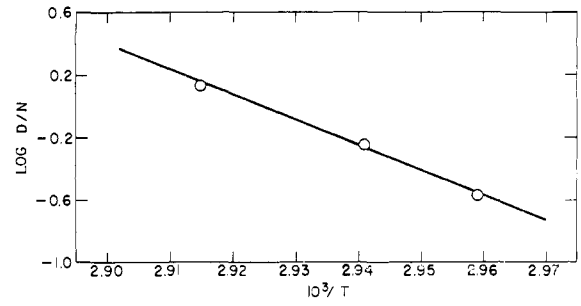



Figure 12. Relationship of log $D/N$ *vs.* $1/T$ indicated by tryptophan N*H* resonances through thermal denaturation in $H_2O$, pH 3.3.

the protein molecule, suggesting that the folding and unfolding processes are cooperative. Since this is the case, the same intensity data can be used to calculate $\Delta H$ and $\Delta S$ for the transition from a conventional log $D/N$ *vs.* $1/T$ plot. The intensity measurements on the tryptophan N*H* resonances at 65, 67, and 70° were considered the most reliable for this purpose since they exhibit assigned, resolved "native" and "denatured" resonances in the same spectrum with absorption integrals sufficiently large to measure with accuracy. Since there is some overlap of resonances IV, V, and the "denatured" resonance, the following relationships were used

$$N = {}^1/_3(R_{\mathrm{I}} + R_{\mathrm{II}} + R_{\mathrm{III}})$$

$$D = {}^1/_6(R_{\mathrm{IV}} + R_{\mathrm{V}} + R_{\mathrm{D}} - 2N)$$

The values of log $D/N$ *vs.* $1/T$ are plotted in Figure 12 and provide values of $\Delta H$ and $\Delta S$ (at pH 3.3, 70°) of 73 kcal $mol^{-1}$ and 215 cal $deg^{-1}$ $mol^{-1}$, respectively. The value of $T_{\mathrm{M}}$, the midpoint of the transition, is 69°. The solid curve in Figure 6 was calculated from these thermodynamic values and can be seen to provide a good "average" representation of the individual data plotted there. Sophianopoulos and Weiss[2] reported a value of $\Delta H$ for the thermal transition at 50° (lower pH) of 64 kcal $mol^{-1}$. The thermodynamic data of Tanford and Aune[7] predict a value of $\Delta H_{70°}$ = 84 kcal $mol^{-1}$ (for the thermal transition N $\rightleftarrows$ X).

Since the pmr spectra characteristic of N and D show no evidence of "exchange averaging" or even of "exchange broadening," lifetimes of these conformations must exceed $5 \times 10^{-3}$ sec (this limit was established from spectra at pH 3.3, 60–70°). However, it must be borne in mind that the spectra of either N or D could derive from either unique structures or time averages of multiple structures. That is, either the native state or the denatured state or both may encompass a multiplicity of conformations undergoing rapid interconversion (lifetimes in individual conformations less than, say, $5 \times 10^{-4}$ sec) among themselves *but not with the other state.*

Various models of protein denaturation-renaturation have been advanced that involve partially folded states between the native and random-coil forms. In particular, two such models, based on statistical mechanical considerations, have been advanced by Poland and Scheraga.[21] There is experimental evidence of intermediate forms in the slow renaturation of ly-

(21) D. Poland and H. A. Scheraga, "Poly-α-Amino acids," G. D. Fasman, Ed., Marcel Dekker, New York, N. Y., 1967, pp 391–497.

sozyme from a denatured form in which the disulfide bonds have been ruptured.[8] When the disulfide bonds are not broken, as mentioned earlier, Sophianopoulos and Weiss[2] found no evidence of transition intermediates in the thermal denaturation of lysozyme. However, on the basis of extensive optical rotation studies, Tanford and coworkers[4-7] have concluded that thermally denatured lysozyme (at least at highly acidic pH) is not a completely extended cross-linked random coil, but retains up to 30% structure that is only removed by 4–5 *M* guanidine hydrochloride. It is crucial, therefore, for the purposes of this investigation to examine the pmr spectra of lysozyme for evidence on whether the individual spectra of the native and denatured states arise from single or multiple structures.

The two models of Poland and Scheraga that involve intermediate states in the folded ⇄ denatured conformational transition of proteins are depicted schematically in Figure 13. Temperature is along the ordinate and the abscissa is some measure of the reduction of the tertiary (and perhaps secondary) structure, *e.g.*, "random-coilness," of the protein. States $S_1$ and $S_n$ would represent, respectively, completely folded and completely unfolded conformations. We can assign chemical shifts $\delta_1^i$, $\delta_2^i$ ... $\delta_n^i$ for the *i*th proton in the protein to these various states. There, of course, is no reason to expect that these chemical shifts will be linear functions of the undefined states between $S_1$ and $S_n$. We may reasonably expect that the magnitudes of the various $\delta_2^i$, $\delta_3^i$ ... $\delta_{n-1}^i$ will fall between those of $\delta_1^i$ and $\delta_n^i$.

It appears that model I can be eliminated immediately for HEW lysozyme on the basis of the pmr results. This model predicts observation of only a single set of resonances with a continuous change over the transition region in chemical shift between shifts characteristic of the native and denatured forms of the protein. This behavior is not observed; rather two sets of resonances are observed and the spectrum of the folded conformation simply decreases in intensity without perturbation in chemical shifts over this region. Model II is similarly rejected since it is difficult to envisage how such a model could give rise to a spectrum for the protein in the thermal transition region essentially identical with that of the enzyme at 35°. Again, assuming interconversion between the states $S_1$ ... $S_n$ to be rapid on the nmr time scale, we would expect a monotonic alteration of chemical shifts from folded to denatured characteristics over the transition range. As has been indicated, such behavior is not observed for HEW lysozyme.

To what extent, then, are the pmr results compatible with a two-state denaturation–renaturation process? We have found no evidence of states other than the denatured and native states and now consider what can be said about the nature of these states. First, we note that the denatured state, as an extended form of the protein, undoubtedly encompasses many configurations of about equal energy and probability. The real question here is whether species containing elements of folded structure also contribute significantly to the cross-linked random-coil state. The four intact disulfide bridges may well promote retention of specific conformations. We have been unable to distinguish

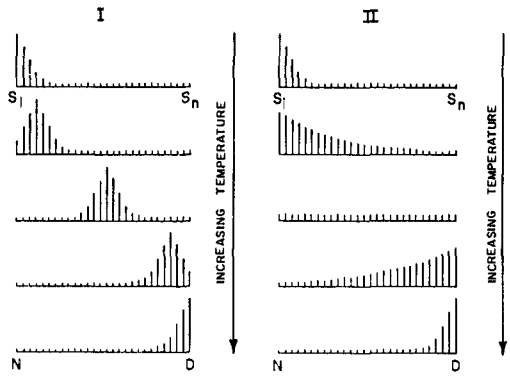



Figure 13. Models for the denaturation–renaturation of proteins (D. Poland and H. A. Scheraga, ref 20).

any differences in the pmr spectra of the side-chain protons of denatured lysozyme whether denaturation was effected by temperature, acid, or guanidine (except expected changes due to changes in ionization of the protein). The spectra indicate that all of the side chains are in an equivalent solvent environment. However, elements of structure may remain (*e.g.*, regions of $\alpha$ helix) in which the side chains are all solvated and which are therefore, indistinguishable by pmr from side chains in extended polypeptide regions. Such structures may account for the differences that Tanford, *et al.*,[5] find between the denatured states achieved by heating and guanidine, respectively. Also, small fractions of partially structured forms (wherein the side chains are not solvated) may exist in fast interconversion with the fully extended forms without noticeably perturbing the pmr spectrum. In summary, pmr spectra indicate that most lysozyme molecules in the denatured state are extended with residue chains in a solvent environment, but do not preclude the possibility that elements of secondary structure or even small amounts of tertiary structure are retained.

The pmr spectra seem more definitive in regard to the native state of lysozyme. In particular, we refer to the observation that positions of resonances in the spectral region from 180 to −140 Hz for the native enzyme are either unchanged or shift only a few hertz from 35 to about 75° where the native spectrum disappears. As noted above, these resonances are very sensitive probes of small conformational changes, and they monitor environments in several regions of native lysozyme. Thus, the virtual absence of a temperature dependence for the positions of these resonances strongly suggests that native lysozyme is best represented by a single conformation rather than a family of conformations undergoing rapid interconversion. If there were multiple conformations of native lysozyme, the ring-current shifted resonances of each contributing to the "exchange-averaged" observed spectrum should be somewhat different. The proportions of the contributions of these multiple forms would vary with temperature (unless the multiple forms have very similar energies) and cause readily observable diplacements of the ring-current shifted resonances. The small temperature-induced chemical-shift displacements that are observed for a few resonances of native lysozyme more likely reflect a small local

conformational change of a single lysozyme structure than a redistribution of a population of multiple forms of lysozyme. For example, small temperature dependences of the tryptophan N*H* protons and the histidine C-2 proton reflect changes in hydrogen bonding of these side chains.

# *Communications to the Editor*

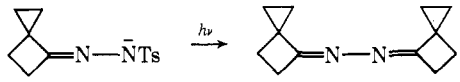
## $\Delta^{1,4}$-Bicyclo[2.2.0]hexene[1]

*Sir:*

$\Delta^{1,4}$-Bicyclo[2.2.0]hexene (I) is one of the more interesting of the unknown small ring alkenes. It is a possible precursor for the theoretically interesting tricyclo[2.2.1.$0^{1,4}$]heptane and tricyclo[2.2.2.$0^{1,4}$]-octane,[2] and for a trans-fused bicyclo[2.2.0]hexane. A knowledge of its strain energy, reactivity, and mode of reaction should contribute to a number of currently important theoretical problems.

The alkene, I, has been elusive. Attempts at dehalogenating 1-bromo-4-chlorobicyclo[2.2.0]hexane (II)[3] with metals have failed, and thus the dehalide is similar to 1,2-dibromocyclopropane.[4] The reaction of II in cyclooctane with sodium–potassium alloy, for example, gives bicyclo[2.2.0]hexane,[5] as well as coupling products of cyclooctane with the bicyclo[2.2.0]-hexane ring system. This is probably a free-radical reaction involving, in one step, a hydrogen transfer from cyclooctane to a bridgehead bicyclo[2.2.0]hexyl radical. The reaction of II with sodium in diglyme as solvent also gave bicyclo[2.2.0]hexane. Finally, the reaction of II with the sodium phenanthrene radical anion[6] gave no low molecular weight products, but rather gave a product containing both the cycloalkane and phenanthrene rings.

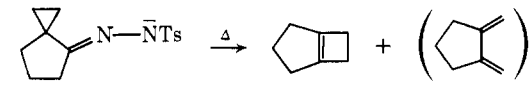
As we have previously reported,[2] the photolysis of the sodium salt III of spiro[2.3]hexanone-4 tosylhydrazone gave the azine IV when diglyme was used as the sol-



III IV

vent. Under these conditions, the reaction was heterogeneous. Dimethyl sulfoxide rendered the reaction homogeneous, but bicyclohexene was not found as a product. The formation of $\Delta^{1,5}$-bicyclo[3.2.0]-heptene from spiro[2.4]heptanone-4 *via* the above reaction, using diglyme as the solvent, has been reported by Kirmse,[7] and this reaction also occurs when dimethyl sulfoxide is used as the solvent.

Although the thermolysis of the sodium salt of spiro-[2.4]heptanone-4 tosylhydrazone has been reported to give a mixture of bicyclo[3.2.0]heptene and 1,2-dimethylenecyclopentane,[7] we have now observed that the reaction, when carried out under high vacuum (in the absence of solvent), leads to a good yield of bicyclo-[3.2.0]heptene and very little of its ring-opened product.



This reaction was applied to III, and the volatile products were collected in a liquid nitrogen cooled trap. As the contents were warmed to room temperature, an exothermic reaction occurred. The products were 1,2-dimethylenecyclobutane and a compound (V) which analyzed by mass spectra and elementary analysis as a dimer. The dimer had a symmetrically tetrasubstituted double bond as shown by the lack of ir absorption in the C=C region, the presence of a band at 1680 $cm^{-1}$ in the Raman spectrum,[8] and the absence of vinyl proton bands in the nmr spectrum. The nmr spectrum had bands at $\tau$ 7.58 (4 H),[9] 7.65–7.95 (8 H), and 8.16 (4 H).

Thermolysis of the dimer V was accomplished in the gas phase at low pressure by passing it through a section of glass tubing heated to about 350°. The product of the thermolysis, VI, had four olefinic protons ($\tau$ 4.65, 5.27) and four allylic protons ($\tau$ 7.27) in an nmr pattern resembling that of 1,2-dimethylenecyclohexane,[10] as well as an nmr band at $\tau$ 7.92 (8 H) which strongly resembled that for the cyclobutane ring protons of tricyclo[3.2.2.$0^{1,5}$]nonane.[11] These data indicate the structures to be those shown below. Therefore, we conclude that the dimer V corresponds to the Diels–Alder adduct of bicyclo[2.2.0]hexene and dimethylenecyclobutane.

The presence of bicyclo[2.2.0]hexene (I) in the reaction mixture was demonstrated by adding cyclopentadiene to the liquid nitrogen cooled reaction product before warming it to room temperature. No dimer V was found under these conditions, but rather, a new

(1) This investigation was supported by the U. S. Army Research Office.

(2) K. B. Wiberg, J. E. Hiatt, and G. Burgmaier, *Tetrahedron Lett.*, 5855 (1968).

(3) K. V. Scherer, Jr., and T. J. Meyers, Abstracts, 155th National Meeting of the American Chemical Society, San Francisco, Calif., April 1968, No. P 180.

(4) K. B. Wiberg and W. J. Bartley, *J. Amer. Chem. Soc.*, **82**, 6375 (1960).

(5) We wish to thank Dr. R. Srinivasan for supplying the nmr spectrum of bicyclo[2.2.0]hexane.

(6) This reagent has been used for the dehalogenation of vicinal dihalides: E. Vogel, H. Kiefer, and W. Roth, *Angew. Chem., Int. Ed. Engl.*, **3**, 442 (1964); R. Ubersax, Ph.D. Thesis, Yale University, 1969.

(7) W. Kirmse and K. H. Pook, *Angew. Chem., Int. Ed. Engl.*, **5**, 594 (1966).

(8) The Raman band is at about the same position (1677 $cm^{-1}$) observed for $\Delta^{1,5}$-bicyclo[3.2.0]heptene (see ref 7). The Raman spectrum was kindly run by Kirkwood Cunningham.

(9) Cyclobutene allylic protons lead to nmr bands at $\tau$ 7.4–7.5 (*cf.* K. B. Wiberg and B. J. Nist, *J. Amer. Chem. Soc.*, **83**, 1226 (1961)).

(10) P. D. Bartlett, A. S. Wingrove, and R. Owyang, *ibid.*, **90**, 6067 (1968).

(11) We wish to thank Professor Philip Eaton for supplying the nmr spectrum for this hydrocarbon. It has the corresponding band at $\tau$ 8.05.