Detection of New Temperature-Dependent Conformational Transition in Lysozyme by Carbon-13 Nuclear Magnetic Resonance Spectroscopy (nuclear Overhauser enhancement/magnetic relaxation)

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ABSTRACT A specific temperature-dependent conformational transition of hen egg-white lysozyme, occurring between 20°C and 30°C in solution, has been detected by 13C-nuclear magnetic resonance spectroscopy. Selective changes in the chemical shifts of aromatic residues, together with differences in the chemical shifts, and nuclear Overhauser enhancement in the carbonyl, carboxyl, and alpha-carbon regions of the spectrum point to the vicinity of subsites D and E as the primary locus of the structural change.

It has been recently shown that several proteins and enzymes can crystallize at temperatures higher than was usually assumed. Typical examples of temperature-dependent crystallization are yeast fatty acid synthetase (1), the Fab′ fragment from mouse IgA myeloma protein (2) and duck egg-white lysozyme II (3). The case of hen egg-white lysozyme has been extensively investigated over the past 2 years (4–6). It has been demonstrated that hen egg-white lysozyme crystals can be obtained between 25°C and 60°C. These high temperature crystals (form B) are orthorhombic whereas, the classical crystals obtained in the temperature range from −36°C to 25°C and thoroughly studied by D. C. Phillips and coworkers (ref. 7 and references therein) are tetragonal (form A).

Moreover, A crystals can convert into B crystals under the sole influence of temperature regardless of pH, ionic strength, protein concentration or nature of the precipitating salt (6). The only requirement for the occurrence of this transition is the presence of the liquid phase. In a typical experiment, A crystals obtained at low temperature are heated in presence of the mother liquor. At 22°C–25°C, A crystals dissolve and the formation of B crystals is observed. A and B crystals are present simultaneously at 25°C but the B form is the only one that exists above 25°C and up to 60°C. B crystals have been shown to be more stable than A crystals and, after dissolution, remain fully active against suspensions of Micrococcus luteus cells.

In the light of these findings, it is reasonable to consider the possibility that the lysozyme molecule undergoes a conformational transition in the temperature range close to 25°C which would account for the existence of the two crystalline forms. The observed dimorphism, could of course be accounted for by a crystal packing transition alone, but a significant conformational rearrangement of individual molecules would surely be reflected in a rearrangement of the lattice.

Our aim, therefore, was to investigate limited specific changes in the three-dimensional arrangement of lysozyme in solution that occur in a rather narrow range of temperature corresponding to the crystalline phase transition.

The structure of lysozyme in solution is known to undergo changes as a function of temperature. The reversible denaturation of lysozyme occurs between 60°C and 75°C, and has been studied extensively by McDonald and W. D. Phillips (8). The existence of two types of fluctuating unfolded forms D1 and D2 has been proposed by Nakaniishi et al. (9) on the basis of a hydrogen–deuterium exchange reaction as followed by infrared absorption. Form D1 would predominate in the temperature range of 25°C–50°C whereas, the amount of D2 form would be the largest between 65°C and 85°C. Viscosity measurements and optical rotation have also been used to document the thermal denaturation of lysozyme (10). All these studies, however, have mainly described large overall structural changes between the two extreme states (native and full denatured) under which the lysozyme molecule can be found below and beyond a temperature of 65°C.

Since the work of Allerhand and his colleagues (11), the potential of carbon-13 nuclear magnetic resonance spectroscopy for detailed study of biopolymers in solution has been clearly established. Carbon-13 NMR spectra of proteins obtained in natural abundance of the carbon-13 isotope potentially contain a considerable amount of information about the organization of the polypeptide backbone and of different side chain groups. The availability of Fourier Transform techniques and of large diameter sample tubes (12–25 mm) makes it possible to obtain a signal-to-noise ratio adequate for observing single carbon resonances in several proteins including hen egg-white lysozyme (12). It is thus possible to follow at a discrete level the behavior of several individual residues or classes of residues when the protein is subjected to various perturbing conditions.

In this study, it has been possible to infer from selective changes in the chemical shifts of single carbon lines and groups of lines, accompanied by changes in relaxation times and nuclear Overhauser enhancement (NOE), that a localized conformational change occurs in the lysozyme molecule in the vicinity of subsites D and E of the active site in the range of temperature from 20°C to 30°C.

Considering that the temperature of in vivo reactions is 40°C in birds, it is possible that the high temperature structure corresponding to the B crystals is more relevant from a biological standpoint than the more accurately known structure corresponding to A crystals.
MATERIALS AND METHODS
Pure samples of hen egg-white lysozyme were crystallized six times. Lysozyme was dissolved in 100% D2O (Diaprep) at a concentration of 180 mg/ml and adjusted to pH 4.75. No correction was made for deuterium effect in the pH reading.

Carbon-13 NMR spectra were recorded at 25.2 MHz on a Varian XL-100-15 spectrometer equipped with a Nicolet model NMR-80 36K computer. The probe contained spinning sample tubes of 12 mm outside diameter. A spectral width of 6000 Hz was used for recording the complete spectrum of lysozyme with a digital resolution of 1.33 Hz. The instrument was modified for increased sensitivity by using a single side band crystal filter on the intermediate frequency signals. The spectra of Figs. 1, 2, 4, and 5 were obtained by collecting free induction decays following 90° radiofrequency pulses for an acquisition time of 1.36 s. The final data were stored in 16K data points and an additional 16K zeroes were added. The entire data table was multiplied by a negative exponential corresponding to 2.0 Hz line broadening. Heteronuclear proton noise decoupling was achieved with a Varian gyrocode Spin Decoupler model V-4421. A Varian temperature controller was used for temperature variations with an accuracy of ±0.5°C.

Spin-lattice relaxation times (T1) for the α-carbon region were determined by the inversion-recovery technique (15). Nuclear Overhauser enhancements were measured by the gated decoupler method (16).

Difference spectra were calculated using Nicolet software. All chemical shifts are reported with respect to an external capillary of tetramethylsilane (TMS).

RESULTS AND DISCUSSION
The spectral changes which permit the inference of a temperature-dependent conformational transition are apparent in the spectra of native lysozyme (Figs. 1 and 2). No significant perturbation was observed in spectra obtained within the temperature range from 16°C to 20°C or within the higher temperature range from 30°C to 42°C. Consequently, only the typical 20°C and 30°C spectra are presented.

Fig. 1 demonstrates a variety of changes throughout the aliphatic region. Most pronounced are those in the alpha (50–65 ppm) and methylene. The difference spectrum of the α-carbon resonances (Fig. 1c) shows a pattern of chemical shift differences that can only reflect a rearrangement of a sizeable number of residues in the molecule. No major variation in side chain mobility can be occurring since the line widths of the high field signals are also similar, indicating that no large changes in the NOE contributions to signal intensity are observed.

The high temperature pattern (30°C) of the α-carbon region has previously been characterized by Allerhand et al. (12). At lower temperature, (20°C), the appearance is different but equally characteristic (Fig. 1b). Because of the overlap in this spectral region, no direct assignment as to what resonances are changing is possible. Nevertheless, it is apparent that the multitude of changes reflect small shifts of a large number of resonances, rather than discrete shifts of a few resonances.

The chemical shifts can be used as a reliable monitor of the conformational state of the protein, and by themselves indicate the occurrence of a conformational transition. Additional information on conformational differences is obtainable from relaxation measurements, notably the longitudinal relaxation time (T1) and the nuclear Overhauser enhancement.

The α-carbon resonance region represents a chemically homogeneous population of atoms. Because the α-carbons in a native protein structure are sterically highly restricted, it is a reasonable approximation to treat them as if they have no internal motion. If this is a valid approximation, then the spectroscopic parameters of this region can be used to de-
termine an overall isotropic rotational correlation time of the molecule as a whole. This has been done in the case of muscle calcium binding protein from carp, and excellent agreement was found with an experimental determination by depolarized light scattering (16).

Lysozyme has an overall rotational correlation time of 8.5 ns at 30°C (17, 18). If the α-carbons are held rigidly in the protein structure, values of $T_1 = 42$ ms, and NOE = 1.2 would be expected. The experimental results ($T_1 = 40$ ms, NOE = 1.3) are in excellent agreement with the predicted values. This is good evidence that the relaxation of the α-carbons is determined by the isotropic tumbling of the molecule as a whole.

At 20°C, depolarized light scattering measurements yield a value of 10 ns for the correlation time (18), a 15% increase from the value at 30°C which is probably due only to the change in the viscosity of water. For this reorientation time, $T_1 = 45$ ms and, NOE = 1.2. The $T_1$ for this case, was measured to be slightly larger than at 30°C, again in good agreement with the predictions of the rigid isotropic model. In addition, the overall NOE of the α-carbon region was slightly reduced as expected. However, the most striking observation of this experiment occurs in the upfield portion of the α-carbon region as observed by NOE measurement. Apparently there is a collection of resonances with a relatively high field chemical shift and a significantly large NOE. This result was reproducible in all respects, and the spectra in Fig. 3 are characteristic of the low temperature state. Again, because of the overlap, the effect cannot be quantitated, but it is apparent that the number of resonances with a large NOE is small. It is not surprising that this small fraction does not alter the appearance of the inversion-recovery $T_1$ plot. As found in a previous study (17) the NOE is a very sensitive function to monitor altered intramolecular motion in proteins.

Thus, the α-carbon data show that at 30°C, all parts of the protein backbone are rigid with a correlation time of about 8.5 ns. At 20°C, a segment of the protein backbone has some segmental motion of a nature which might have an apparent correlation time up to one order of magnitude different than the overall tumbling time. This change in resonance behavior even without detailed interpretation also indicates a difference in the conformational state of the protein between the two temperatures.

The downfield portion of the spectrum (165-185 ppm) shown in Fig. 2 and corresponding to the carbonyl and carboxyl resonances likewise undergoes many changes in chemical shifts and line intensities as a result of the temperature increase. Interpretation of intensity changes in these resonances is more difficult than in the case of carbons bearing hydrogens because the relaxation mechanism of the former is not dominated by dipolar interactions with protons. However, the chemical shift changes which are observed strongly suggest a rearrangement of the secondary structure.

The changes in the aromatic region of the spectrum (100-160 ppm) (Fig. 2) are the easiest to interpret since several single carbon resonances can be resolved in spite of the low signal-to-noise ratio attained in this region.

Lysozyme contains six tryptophan residues whose γ-carbon resonances have been identified and partially assigned by Allerhand et al. (12, 14) in the 107-115 ppm region of the 30°C spectrum.

At least, two specific changes are apparent from a comparison of the γ-carbon signals of Trp at 20°C and 30°C (Fig. 4). With the increase in temperature, the downfield resonance (112.3 ppm), assigned to Trp 108, shifts upfield by 5 Hz. The upfield resonance of the upfield doublet (108.0 ppm), assigned to either Trp 111 or Trp 28, exhibits a 10 Hz downfield shift. This appearance of selective shifts of side chain resonances

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**Fig. 3.** NOE measurement of the alpha carbons of lysozyme. Acquisition time equals 0.34 s. Delay with decoupler turned off = 0.34 s. 10,000 transients were collected and multiplied by a negative exponential corresponding to 4 Hz line broadening. The lower spectra display the intensity (I) achieved without NOE effect. The contribution of NOE to the signal intensity (I$_s$ + I$_s$) is shown on the upper curve.

**Fig. 4.** Expansion of the spectral region of Fig. 2 corresponding to the resonances of the γ-carbons of the six tryptophan residues. (a) 30°C, (b) 20°C.
allows the localization of the conformational change. Trp 108, which forms a hydrogen bond with Gln 57, as well as Trp 28 or Trp 111, which is hydrogen-bonded to Asn 27, lie in a close proximity according to the low temperature crystal structure (7). The changes in the spatial arrangement at 30°C which are reflected in the observed shifts of the Trp resonances, can thus be localized on one edge of the cleft of the lysozyme molecule.

The three signals corresponding to the zeta-carbon atoms of the three tyrosine residues of lysozyme are shown on Fig. 5. When increasing the temperature from 20°C to 30°C, the upfield signal (153.8 ppm), tentatively assigned to Tyr 53 remains unaffected whereas the low field signal corresponding to Tyr 20 or 23 exhibits a 4.5 Hz downfield shift. This perturbation of one Tyr residue points to the same region of lysozyme as the tryptophan shifts.

Other changes in the chemical shifts are consistent with the notion that this portion of the edge of the cleft is the main site of the conformational change. At 30°C, upfield shifts (Fig. 2) of the guanido-carbon atoms of some of the arginines are apparent (157.4 ppm) and we have previously reported important changes in the β-methyl carbons of alanines. Residues 112 and 114 are arginine, whereas, alanine is present at position 107 and 110.

All of the foregoing evidence indicates that lysozyme undergoes a conformational transition at pH 4.75 in the temperature range from 20°C to 30°C. The transition is totally reversible, and affects a limited but substantial part of the molecule; the major changes point to the portion corresponding to subsites D and E and possibly involving the α-helix between residues 109 and 115. It is noteworthy that this thermal transition has not been detected by microcalorimetric methods (19). This study, clearly confirms carbon-13 magnetic resonance spectroscopy as a powerful investigative tool in protein conformation analysis. Whether or not the described temperature-dependent conformational transition in solution is related to the crystalline phase transition remains now to be demonstrated. If so, it might be necessary to reexamine some of the current concepts of the proposed mechanism of action of lysozyme, most of which are derived from studies of the low temperature crystal form.

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