Evidence for a New Cooperative Transition in Native Lysozyme from Temperature-Dependent Raman Optical Activity

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On account of its special sensitivity to conformational mobility in chiral molecular structure, vibrational Raman optical activity has revealed what appears to be a new type of sharp cooperative transition in a native protein, possibly related to its function, from measurements on hen egg white lysozyme in the temperature range 2−50 °C. It occurs at ∼12 °C and involves the loss of residual mobility in tertiary loop and secondary structure and, in tryptophan side groups, in both domains of the protein including the active site region. No significant conformational change appears to be involved. Since differential scanning calorimetry failed to reveal any latent heat, it does not appear to be a simple first-order phase transition.

Introduction

It has been appreciated for some time that proteins are dynamic systems which experience a wide range of motions from fast local oscillations about bonds to slower cooperative motions of segments of the overall structure.[1,2] Among the most important sources of experimental information about the details of protein dynamics at the atomic level are X-ray structure B-factors and NMR spectroscopy.[2] However, there is still a dearth of experimental information on these motions and their relationship to protein stability, folding, and function, which are topics of great current interest, both fundamental and practical. Theories of protein dynamics have been formulated mostly within the established frameworks of thermodynamics and molecular mechanics,[1,2] but more recently methods from the physics of complex systems[3] including glasses[4] and spin glasses[5,6] have been applied to the problem but as yet remain largely untested experimentally.

A promising experimental technique for such studies is Raman optical activity (ROA), which measures vibrational optical activity by means of a small difference in the intensity of Raman scattering from chiral molecules in right and left circularly polarized incident laser light.[7−11] Recent advances in instrumentation[12,13] have now rendered proteins in aqueous solution accessible to ROA measurements, which provide an incisive new probe of secondary and tertiary structure and dynamics.[14−18] Here we show that ROA spectra of native hen egg white lysozyme measured over a range of temperatures, in conjunction with recent NMR data,[19,20] provide evidence for a previously undetected cooperative transition between mobile and rigid structures. In an earlier paper[15] we presented an ROA study of lysozyme under various conditions which provides a background for the present study. However, the ROA spectra shown here are of higher quality since they were measured on an upgraded instrument.

Experiment

Hen egg white lysozyme was obtained from Sigma (product number L6876) and used without further purification. Gel filtration and SDS gel electrophoresis gave results consistent with Sigma's estimate of 95% protein content. The studies were performed in 0.1 M sodium acetate buffer at pH 5.4 prepared using Analar grade reagents and distilled, deionized water. Protein solutions at ∼70 mg/mL, prepared in small glass sample tubes, were mixed with a little activated charcoal (pharmaceutical grade) to remove traces of fluorescing impurities and centrifuged. Solutions were subsequently filtered through Millipore GV4 (0.22 μm) filters directly into quartz microfluorescence cells, which were again centrifuged gently (to clear any remaining dust particles) prior to mounting in the ROA instrument. Residual fluorescence from remaining traces of impurities was allowed to "burn down" by leaving the sample to equilibrate for several hours in the laser beam before acquiring ROA data. This procedure, which is standard in Raman spectroscopy of proteins,[21] did not appear to harm our samples because their Raman spectra remained unchanged (denaturation is readily detected in Raman spectra). Our confidence in the integrity of the lysozyme sample was reinforced by finding that it retained its full enzymatic activity (as measured by the standard Sigma assay based on cell wall hydrolysis of *Micrococcus lysodeikticus*) even after exposure to the laser beam for several days.

The instrument used for the ROA measurements utilized backscattering, which is essential for aqueous solution samples of biopolymers, and employs a single-grating stigmatic spectrograph based on a novel holographic transmission diffraction grating fitted with a back-thinned CCD camera as detector and a holographic notch filter to block the Rayleigh line.[12,14] ROA is measured by synchronizing the spectral acquisition with an electrooptic modulator used to switch the polarization of the incident argon ion laser beam between right and left circular at a suitable rate. The ROA spectra are presented in the form of a raw circular intensity difference $I^R - I^L$, where I^R and I^L are the Raman-scattered intensities in right and left circularly polarized incident light. The conventional Raman intensities are presented as a corresponding circular intensity sum $I^R + I^L$. The experimental conditions were as follows: laser wavelength 514.5 nm; laser power at the sample ∼700 mW; spectral bandwidth (fwhh) ∼12 cm^{-1}; recording time 10 h for each ROA spectrum.

The temperatures were established by blowing dry air over the sample using an FTS Systems Model TC-84 Air Jet Crystal Cooler. The ROA spectrum at the lowest temperature, 2 °C, was measured first with the sample being kept previously overnight in the refrigerator at 2 °C and then left for several hours in the cold air stream to allow it to equilibrate fully in the laser beam before commencing data acquisition. Subsequent ROA spectra were acquired after the sample was left to equilibrate for several hours *in situ* at each new successively higher temperature. The quoted temperatures will be underes-

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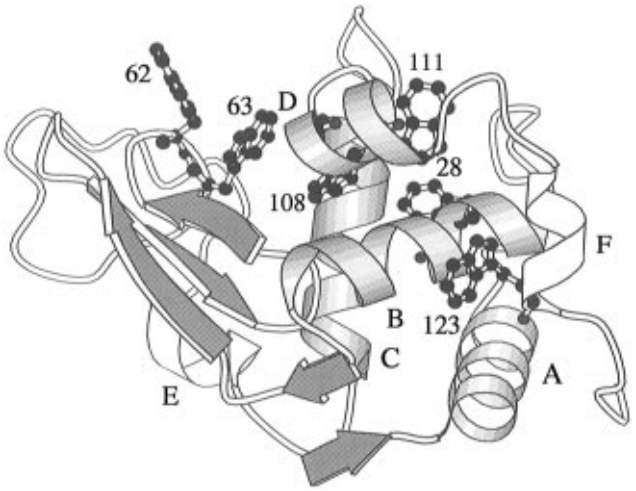

Figure 1. Schematic view of the polypeptide backbone of hen egg white lysozyme, together with the six tryptophan residues, illustrating the α- and β-domains and the active site region formed at the interface between them.[23] The figure was prepared with the program MOLSCRIPT.[40]

timates by several degrees of the true sample temperatures along the laser beam inside the cell since measurements were taken at the outside wall of the cell.

Results and Discussion

To set the scene, we show the structure of lysozyme in Figure 1 followed by the room temperature Raman and ROA spectra of lysozyme, measured at 20 °C, with the different regions marked as the top pair in Figure 2. A detailed discussion of the origins of the various ROA bands can be found in ref 15, but we note here that the backbone $C_\alpha-C$ and $C_\alpha-N$ skeletal stretch region is dominated by secondary structure bands, that the extended amide III region contains bands characteristic of loops and turns, as well as secondary structure, originating in coupled backbone N−H and $C_\alpha-H$ deformations together with the $C_\alpha-N$ stretch and is particularly sensitive to the Ramachandran ϕ angle, and that the amide I region which arises predominantly from the C═O stretch contains a superposition of bands from both secondary and loop structure. Characteristic α-helix ROA signatures include positive intensity in the range ∼880−960 cm^{-1}, the couplet with a small negative component at ∼1095 cm^{-1} and a large positive component at ∼1120 cm^{-1}, and the strong sharp positive band at ∼1296 cm^{-1} with a negative shoulder at ∼1265 cm^{-1}. The broad positive ROA band centered at ∼1000 cm^{-1} probably originates in β-sheet vibrations, although contributions from aromatic side groups cannot be ruled out. Clear signatures of rigid loop structure in the extended amide III region include the sharp negative and positive bands at ∼1237 and 1340 cm^{-1}, respectively, associated with different types of local order. Side group ROA bands can also be identified: for example, tryptophan vibrations are responsible for the strong Raman band at ∼1555 cm^{-1} associated with a strong positive ROA band and for the weak Raman band at ∼1580 cm^{-1} associated with weaker negative ROA, and the strong Raman band at ∼1451 cm^{-1} which originates in CH_2 and CH_3 deformations, with a contribution from tryptophan to the ∼1420 cm^{-1} shoulder, is associated with an ROA couplet negative at low wavenumber and positive at high.

We have previously observed that, when the protein is unfolded by reducing all four disulfide bonds and maintaining low pH, most of the ROA bands shown by native lysozyme disappear and the spectrum becomes dominated by a large extended amide III ROA couplet which (since the time scale of Raman scattering process, $\sim10^{-14}$ s, is much shorter than that

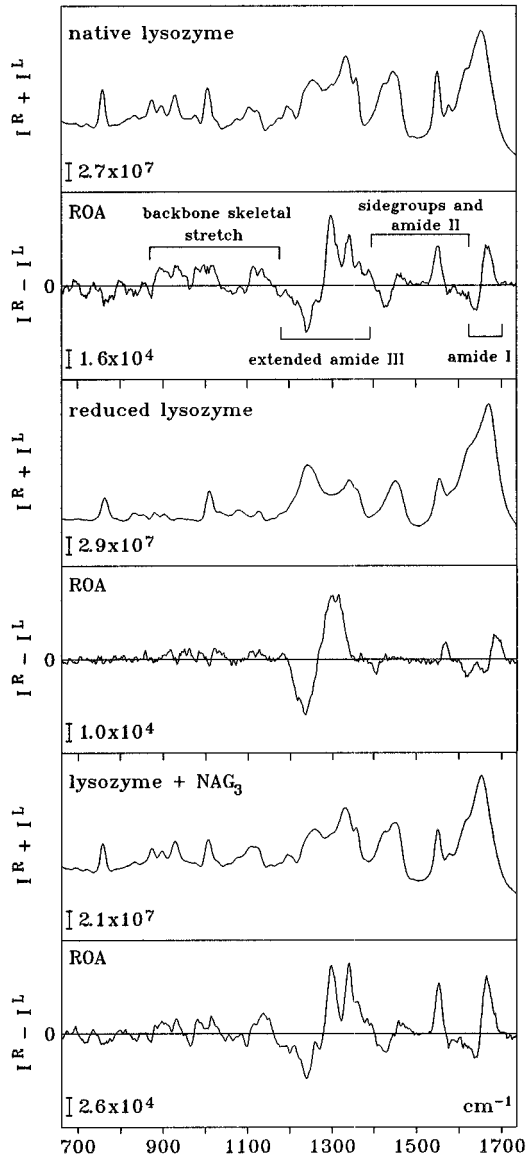

Figure 2. Backscattered Raman ($I^R + I^L$) and ROA ($I^R - I^L$) spectra of native hen egg white lysozyme in acetate buffer at pH 5.4 (top pair), reduced hen egg white lysozyme in citrate buffer at pH 2.0 (middle pair), and lysozyme bound to the trimer of *N*-acetylglucosamine in acetate buffer at pH 4.5 (bottom pair). All were measured at 20 °C.

of typical conformational changes) originates in a superposition of "snapshot" amide III signals from the enormous number of distinct conformers present in the unfolded state and suggested that a similar couplet which underlies the amide III region in the ROA spectra of native lysozyme (and not to be confused with the amide III bands originating in rigid secondary and loop structure) might originate in heteropolypeptide backbone structure with the same type of conformational heterogeneity and associated mobility as that of an unfolded protein.[15] New higher-quality Raman and ROA spectra of reduced lysozyme, measured at 20 °C, are shown as the middle pair in Figure 2. Further studies suggest that the sharp negative peak at ∼1237 cm^{-1} and the two sharp positive peaks at ∼1300 and 1314 cm^{-1} reflect the propensities of individual residues in the mobile heteropolypeptide to adopt Ramachandran ϕ, ψ angles similar to those found in polyproline II helix, α-helix, and β-structures, respectively.[18]

ROA spectra of native lysozyme were measured at 2, 8, 12, 15, 20, and 50 °C, and these are displayed in Figure 3. As can be seen, the underlying amide III ROA couplet collapses to zero over quite a small temperature range below 15 °C with a number of the other ROA bands becoming much sharper and more

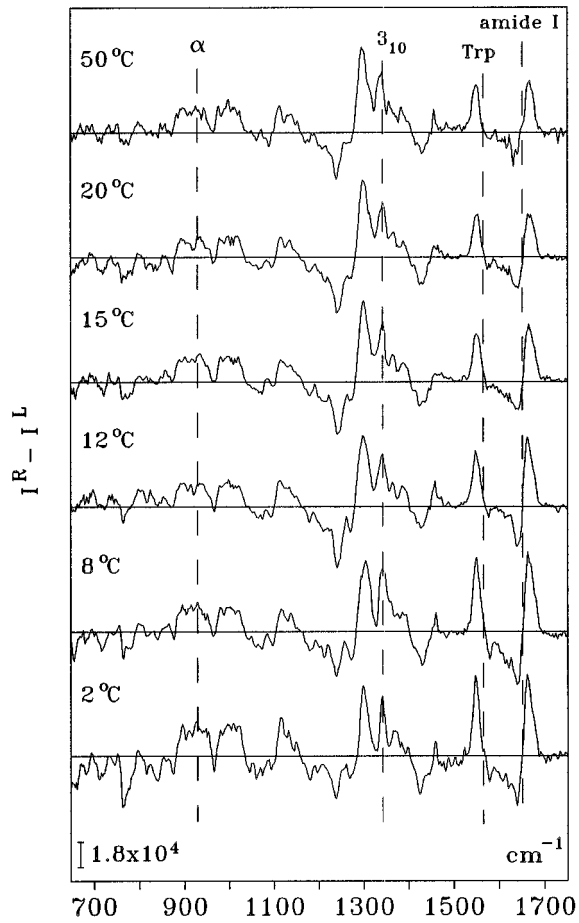

Figure 3. Backscattered ROA spectra of native hen egg white lysozyme in acetate buffer at pH 5.4 measured over the temperature range 2−50 °C which demonstrate the collapse of the underlying extended amide III couplet from mobile structure and the concomitant boost of the signatures of ordered peptide backbone elements and tryptophan side groups at low temperature. The parent Raman spectra are all very similar to that at 20 °C shown at the top of Figure 2.

intense, suggesting that the associated disordered mobile structure has coalesced into rigid structure with well-defined conformational elements. Remarkably, apart from a gradual small improvement in resolution with decreasing temperature, no significant concomitant changes are perceptible in the parent conventional Raman spectra (not shown). The 50 °C ROA spectrum is very similar to that at 20 °C, showing that the transition is essentially complete at room temperature with no further changes occurring up to near the onset of thermal denaturation. Can we deduce what has happened to the protein from the observed changes in the ROA spectral details?

The positive ∼1340 cm^{-1} ROA band, which has increased by ∼40% on reducing the temperature from 15 to 2 °C, is of particular interest since evidence is accumulating that the local order associated with the corresponding rigid loop structure is that of 3_{10}-helix.[16,17,22] The X-ray crystal structure of hen egg white lysozyme[23] contains two stretches of 3_{10}-helix, namely residues 79−84 and 118−124 (E and F respectively in Figure 1), but interestingly two-dimensional ^{1}H NMR studies[19] did not detect a well-defined conformation for residues 79−84 in the aqueous solution structure at 35 °C although this might be attributed to insufficient experimental data for this region. Recent ^{15}N NMR relaxation studies[20] have shown that residues 83−88 have low order parameters (residue 85 is especially low) in solution despite having crystal B-factors no higher than the average. All this suggests that the increase in the positive ∼1340 cm^{-1} ROA band intensity is monitoring a coalescence of this region into the nascent 3_{10}-helix on lowering the temperature. This idea is supported by our earlier observation[15] that the intensity of the positive ∼1340 cm^{-1} ROA band also increases by ∼40% when lysozyme is bound to the trimer of *N*-acetylglucosamine (NAG_3) which induces a more rigid protein structure (new higher-quality Raman and ROA spectra of the lysozyme−NAG_3 complex, measured at 20 °C, are shown as the bottom pair in Figure 2.

Furthermore, the intensities of the α-helix and *β*-sheet ROA bands in the backbone skeletal stretch region have increased by ∼50% and that of the amide I couplet by nearly 100% on reducing the temperature from 15 to 2 °C. The ^{15}NMR data[20] show that one of the four stretches of α-helix, namely helix D in Figure 1 based on residues 108−115 and comprising just under 20% of the total α-helix content, is less ordered with significantly lower hydrogen bond energy per residue than the other three stretches of α-helix. Perhaps ROA is monitoring the locking of a number of hydrogen bonds (as part of a cooperative nonenthalpic process involving other regions of the protein, *vide infra*) in this stretch of α-helix, together with segments from other stretches, at lower temperature which generates the fully-defined rigid α-helix necessary to show the characteristic ROA signatures. Similar arguments obtain for some of the *β*-sheet structure.

There are also interesting changes in side group ROA bands on lowering the temperature, the most dramatic being the sharpening and almost doubling of the tryptophan couplet positive at ∼1555 cm^{-1} and negative at ∼1580 cm^{-1}. Since ROA probes the local geometry of the tryptophan residues relative to the peptide backbone *via* coupling of the vibrational coordinates of the residue with those of the connecting bonds, this suggests a significant increase in rigidity of several of the six typtophan side groups relative to the backbone. Likely candidates include tryptophans 62 and 63, the indole hydrogens of which are not hydrogen bonded in the crystal, with tryptophan 62 having an especially low solution order parameter according to ^{15}N NMR together with a high crystal B-factor.[20] These, together with tryptophan 108, are implicated in the catalytic action of lysozyme. Since the stretch of 3_{10}-helix 79−84 (E) suggested to be mobile at room temperature is closely linked to tryptophans 62 and 63 by a disulfide bond 64 ↔ 80, its coalescence into rigid structure on reducing the temperature might well be mechanically coupled to the increase in rigidity of these tryptophans. This idea gains currency from the fact that 3_{10}-helix E forms part of the hinge region associated with the opening and closing of the active site cleft, and tryptophans 28, 62, 63, and 108 play an essential role in the hinge-bending normal mode.[2]

We detected no changes in the tryptophan fluorescence characteristics (wavelength and intensity) of lysozyme on reducing the temperature to 2 °C, and none were seen in the near UV CD spectrum, which suggests that the transition observed *via* ROA does not involve significant changes in the global environments of the tryptophans. However, a change was seen in the far UV CD spectrum in the form of a small increase (∼10%) in the negative α-helix band at 208 nm accompanied by a shift of 1 nm to lower wavelength. An unsatisfactory feature of these measurements is that it was not possible to make them at concentrations approaching 75 mg/mL as used for the ROA measurements (concentrations of 0.075 and 3 mg/mL, respectively, were used for the fluorescence and the UV CD).

Even more striking than the absence of any fluorescence or near UV CD signatures of a transition was the absence of any sign of a thermal transition from differential scanning calorimetry (DSC) measurements. This time it was possible to use the same concentration as that for the ROA measurements. As-

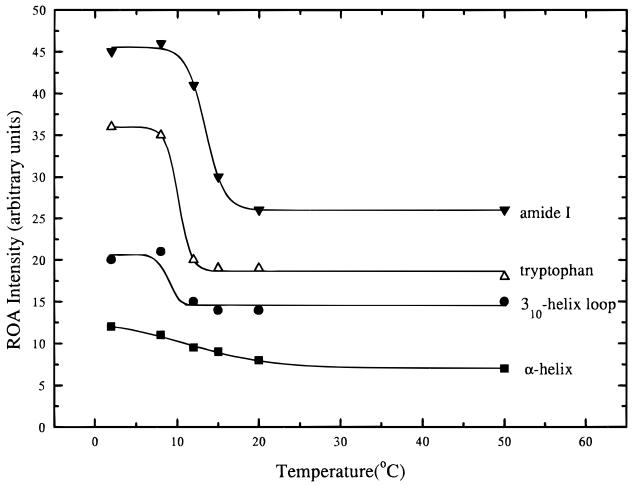

Figure 4. Change of ROA intensity with temperature for the amide I and tryptophan couplets, the 3_{10}-helix loop band at ~1340 cm^{-1}, and the α-helix band at ~931 cm^{-1}. The ROA intensities were measured as background-corrected peak heights for the isolated 3_{10}-helix loop and α-helix bands and as the sum of the amplitudes of the positive and negative peak heights for the amide I and tryptophan couplets. All four curves display the sigmoidal behavior usually associated with cooperativity.

suming a first-order transition, the signal qualities of both the amide I and tryptophan ROA couplets are sufficiently good to use their changes with temperature to construct reasonably straight-line van't Hoff plots of ln K versus $1/T$, where K is the associated equilibrium constant. This leads to an enthalpy change ΔH ~90 kcal mol^{-1} with a transition temperature of ~12 °C. Such a large latent heat should be easily detectable by DSC, so its absence indicates that the putative transition is not first order and hence that the van't Hoff analysis is inappropriate. However, the plots of intensity versus temperature for these two ROA couplets, together with the plots for the 3_{10}-helix ROA band at ~1340 cm^{-1} and (to a lesser extent) the α-helix ROA band at ~931 cm^{-1}, are clearly sigmoidal (Figure 4) which suggests that the transition is nonetheless cooperative.[24]

From the rather high protein concentrations used for the ROA measurements, changes in protein aggregation with temperature might be suspected to be responsible for the transition. We were able to monitor changes in aggregation *in situ* by observing the intensity of the Rayleigh line on the ROA instrument but detected no significant change over the temperature range 0−20 °C.

A possible explanation for the special sensitivity of ROA to mobility that eludes other probes of protein structure emerges from a consideration of the dependence of ROA intensity on torsion angles. Many prominent ROA signals of proteins appear to originate in short-range vibrational coupling, and for these a simple two-group model, in which two achiral axially-symmetric groups or bonds are held together in a twisted chiral structure,[7] provides useful insight. In the special case where the axes of the two bonds are perpendicular to the connecting bond, the ROA intensities $I^R - I^L$ generated by idealized normal modes corresponding to the symmetric (+) and antisymmetric (−) combinations of the local stretch coordinates of the two bonds are proportional to $\pm\sin 2\theta$, where θ is the torsion angle, whereas for idealized normal modes corresponding to the deformations of the angles between the two bonds and the connecting bond, the ROA intensities are proportional to $\mp\sin\theta$. Application of this model to the many two-group structures that can be identified in the polypeptide backbone ($H-C_\alpha-N-H$, $C_\alpha-C(O)-N-C_\alpha$, etc) with subsequent averaging over the range of ϕ, ψ angles encompassed by mobile structure appears to provide a qualitative understanding of the ROA spectrum of reduced lysozyme in particular and of mobile heteropolypeptide structure in general.[18] Essentially, then, the exquisite sensitivity of ROA to transitions involving loss of conformational mobility of the heteropolypeptide backbone is a direct consequence of its dependence on absolute chirality which leads to cancellation of contributions from enantiomeric structures (such as two-group structures with equal and opposite torsion angles), whereas contributions to observables which are "blind" to chirality, such as the conventional Raman band intensities, are additive. Far UV CD is also able to monitor such transitions, but less dramatically than ROA due to its primary dependence on electronic rather than nuclear motion, because it too is sensitive to absolute chirality.

Our observations are reminiscent of the glasslike transitions that have been reported previously in proteins, but these were at much lower temperature and were not in aqueous solution.[25] However, a recent X-ray study of hen egg white lysozyme reported a much smaller number (50%) of ordered water molecules in the nearest hydration shell in the crystal structure at 280 K than in either the 295 or 250 K structures,[26] and it is intriguing that 280 K is close to the temperature of the transition reported here. But any link appears to be tenuous because our measurements were performed in aqueous solution where the protein is fully hydrated.

Since both local and global mobilities are thought to be central to the function of enzymes,[1,2] the residual mobility in lysozyme at physiological temperatures revealed by NMR and ROA in parts of the α- and β-domains, including the active site region and residues involved in the hinge-bending motion, might be related to its activity. This brings to mind the work from over three decades ago[27] on sharp changes at certain temperatures in the slopes of the Arrhenius plots for some enzyme reactions, but to our knowledge no such sharp change in catalytic activity has been reported for lysozyme. One study in particular, on D-amino acid oxidase,[27] attracted some controversy because accompanying changes in fluorescence and other parameters were observed but DSC revealed no latent heat[28] from which it was concluded that, whatever the associated process taking place in the protein was, it did not involve a significant conformational change.[28,29] This conclusion is consistent with that of the present work since the cooperative changes in mobility detected by ROA do not appear to be accompanied by a significant change in protein conformation.

Concluding Remarks

Given the potential importance of the new transition revealed by ROA in native lysozyme, it would be reassuring if its existence could be confirmed using an independent method. Far UV CD seems a promising candidate: although not as sensitive as ROA to changes in mobility, as mentioned above a small change was seen in the α-helix region, but unfortunately insufficient time was available on the service instrument used for a detailed study to be made. Hopefully our results will encourage a group with a dedicated instrument to perform a more thorough UV CD study. Another possibility is vibrational circular dichroism (VCD), the infrared version of vibrational optical activity, which provides information about protein secondary structure from amide I vibrations.[30] In view of the possible functional significance of the transition and the earlier inconclusive work on sharp changes in Arrhenius plots, it might also be worthwhile to search for a coincident change in the catalytic activity of lysozyme, or some other suitable enzyme, and its ROA (or perhaps UV CD or VCD) spectrum as a function of temperature.

Further evidence that the transition is not first order comes from the fact that it is only monitored by the ROA band

intensities, which are pseudoscalar quantities, and not by the parent Raman band intensities, which are scalar quantities. This suggests that the associated order parameter has the symmetry characteristics of a pseudoscalar (like the order parameter associated with the development of the tertiary fold in acid molten globule α-lactalbumin[17]) in which case, within the Landau theory of phase transitions, the cubic terms in the expansion of the free energy in the order parameter near the transition temperature that are necessary for a first-order transition[31] vanish because a pseudoscalar cannot support a cubic invariant.

Assuming that our ROA observations reflect a genuine new transition, it is interesting to speculate on the nature of the physics that might be involved. Since the transition appears not to be first order, it might be a continuous order−disorder phase transition[31] or perhaps a glass or even a spin glass transition between adjacent tiers of conformational substates.[3,5,6] A hint of the metastability characteristic of protein glass states[32] (meaning that the state of the system below the glass temperature depends on its history and that the system is trapped in a substate with an energy above the lowest energy) is provided by the fact that occasionally either the transition was not observed or the signals were much smaller than usual on reducing the temperature (when the sample was not allowed to equilibrate for long enough at the new temperature, for example). The same characteristics were found in the far UV CD studies. From the sharpness of the transition we should also keep in mind the rather unusual heteropolymer transition predicted by Shakhnovich and Gutin[33,34] which combines the features of first- and second-order transitions in that there is a jump in the order parameter but no latent heat. Also, in view of the presence of hydrogen-bonded networks with cooperativity around the many rings embracing both backbone and side groups,[35] there might be a connection with ferroelectric phase transitions of the order−disorder type in crystals involving quenching of cooperative proton tunneling,[36] perhaps coupled with a displacive transition associated with low-frequency modes of vibration of the peptide backbone (such as the hinge-bending mode). Support for the possibility of proton tunneling in proteins comes from recent inelastic neutron-scattering studies of the vibrations of hydrogen bonds in crystalline amides such as *N*-methylacetamide and polyglycine I which demonstrated that the proton is delocalized between two equivalent sites in each bond and experiences a symmetric double-minimum potential;[37] also, hydrogen tunneling can make significant contributions to enzymatic reaction rates under biologically relevant conditions.[38] In aqueous solution interactions between protons and water molecules increase the effective proton mass which suppresses the tunneling,[39] but this problem does not arise if correlated proton tunneling in proteins is confined to the hydrophobic core.

Whatever the detailed mechanism of the putative transition turns out to be, the ROA observations reported here appear to support the view that an individual protein molecule is like a peculiar heterogeneous glass with regions of higher and lower vitrification temperature[4] and provide new experimental data to test ideas such as quantum tunneling and the spin glass analogy for understanding the physics of complex biomolecules.

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