Carbon-13 NMR Relaxation Studies Demonstrate an Inverse Temperature Transition in the Elastin Polypentapeptide†

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ABSTRACT: Carbon-13 NMR longitudinal relaxation time and line-width studies are reported on the coacervate concentration (about 60% water by weight) of singly carbonyl carbon enriched polypentapeptides of elastin: specifically, (L-Val^1-L-[1-^{13}C]Pro^2-Gly^3-L-Val^4-Gly^5)$_n$ and (L-Val^1-L-Pro^2-Gly^3-L-Val^4-[1-^{13}C]Gly^5)$_n$. On raising the temperature from 10 to 25 °C and from 40 to 70 °C, carbonyl mobility increases, but over the temperature interval from 25 to 40 °C, the mobility decreases. The results characterize an inverse temperature transition in the most fundamental sense of temperature being a measure of molecular motion. This transition in the state of the polypentapeptide indicates an increase in order of polypeptide on raising the temperature from 25 °C to physiological temperature. This fundamental NMR characterization corresponds with the results of numerous other physical methods, e.g., circular dichroism, dielectric relaxation, and electron microscopy, that correspondingly indicate an increase in order of the polypentapeptide both intramolecularly and intermolecularly for the same temperature increase from 25 to 40 °C. Significantly with respect to elastomeric function, thermoelasticity studies on γ-irradiation cross-linked polypentapeptide coacervate show a dramatic increase in elastomeric force over the same interval that is here characterized by NMR as an inverse temperature transition. The temperature dependence of mobility above 40 °C indicates an activation energy of the order of 1.2 kcal/mol, which is the magnitude of barrier expected for elasticity.

Inverse temperature transitions, when observed, are commonly considered in terms of a structural change with temperature; usually, physical evidence is presented that a molecular structure increases its order with increasing temperature, and it is then argued, on the basis of the Second Law of Thermodynamics, that clathrate-like water surrounding hydrophobic groups becomes less ordered bulk water as the hydrophobic groups associate (Kauzmann, 1959; Tanford, 1973). The structure of interest is often a polypeptide or protein in which case the hydrophobic groups are the hydrophobic side chains of the constituent amino acid residues. By this process the order of the complete system, water and polypeptide, decreases with increasing temperature, but the order of the polypeptide alone increases as the temperature is increased through the transition.

Since temperature is a measure of molecular motion, a related fundamental statement can be that molecular motion increases with temperature, and an inverse temperature transition would then be a transition in which molecular motion decreased with increasing temperature. This has been demonstrated between 60 and 70 °C for the carbonyl carbons of the polytetrapeptide of elastin, (L-Val^1-L-Pro^2-Gly^3-Gly^4)$_n$, in relatively dilute aqueous solution (Urry et al., 1978). In the present effort carbon-13 nuclear magnetic resonance longitudinal relaxation time (T_1) and line-width studies are reported for carbonyl carbons of the polypentapeptide of elastin, (L-Val^1-L-Pro^2-Gly^3-L-Val^4-Gly^5)$_n$, in a state of direct relevance to elastomeric function at physiological temperatures. The particular interest in the carbonyl carbons is as a monitor of the peptide moiety, the motional processes of which have been considered in relation to the mechanism of elasticity (Urry, 1984; Urry et al., 1982).

The polypentapeptide (PPP) of elastin is soluble in water in all proportions below 20 °C. When the temperature is raised, the samples begin to become cloudy at 25 °C. This temperature-induced association continues to about 40 °C. On being allowed to stand, for solutions that are more than 60% water by weight, the aggregates settle to form a dense viscoelastic phase. The dense phase is called a coacervate, and the process of PPP coacervation is entirely reversible. Of particular interest is that chemical (Urry et al., 1976) and γ-irradiation cross-linking (Urry et al., 1985a) can be used to demonstrate that the cross-linked polypentapeptide coacervate is elastomeric and capable of an elastic modulus similar to that of the natural fibrous elastin within which it is a component. Furthermore, thermoelasticity studies on the γ-irradiation cross-linked polypentapeptide coacervate show the elastomeric force to increase dramatically over the same temperature range for which coacervation occurs (Urry et al., 1984), and dielectric relaxation studies show the development of an intense localized transition (Henze & Urry, 1985), which parallels in its intensity the development of elastomeric force (Urry et al., 1984).

In this paper, L-[1-^{13}C]Pro^2-PPP and [1-^{13}C]Gly^5-PPP are studied at the 30 °C coacervate concentration, which is about 60% water 40% polypentapeptide by weight. In both T_1 and line-width studies, there is observed an abrupt transition beginning at about 25 °C and ending at about 37 °C that parallels the elastomeric force and dielectric relaxation dependence on temperature. The T_1-derived carbonyl rotational correlation time (τ_c) for the Pro^2 carbonyl shows an abrupt decrease in mobility commencing near 25 °C and ending near 37 °C. In the 40–70 °C temperature range, a plot of log τ_c vs. T^{-1} is nearly linear and indicates an activation energy of 1.2 kcal/mol. The correlation times derived from the NMR relaxation studies, assuming isotropic motion and dipole–dipole interactions, are compared with the correlation time obtained for the same polypentapeptide coacervate system following the same inverse temperature transition by means of dielectric

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relaxation studies in the 1–1000-MHz frequency range (Henze & Urry, 1985). Since the dielectric relaxation derived estimates of correlation times depend only on fitting a relatively simple expression to the experimental curve, comparison with NMR-derived correlation times, the calculation of which is more complex, is of interest.

Materials and Methods

The two polymers L-[1-^{13}C]Pro^2-PPP and [1-^{13}C]Gly^5-PPP were synthesized with the monomer sequence as Gly^3-L-Val^4-Gly^5-L-Val^1-L-Pro^2 instead of L-Val-L-Pro-Gly-L-Val-Gly for the reasons described earlier (Urry & Prasad, 1985).

L-[1-^{13}C]Pro^2-PPP. L-[1-^{13}C]Proline (99% enriched, Cambridge Isotope Laboratories, Cambridge, MA) methyl ester was prepared (Brenner & Huber, 1953) by using thionyl chloride and coupled with Boc-L-Val-OH by means of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDCI) (Sheehan et al., 1965) and 1-hydroxybenzotriazole (HOBt) (König & Geiger, 1970). The dipeptide methyl ester obtained was deblocked and reacted with Boc-Gly-L-Val-Gly-OH (Prasad et al., 1985) to yield Boc-Gly-L-Val-Gly-L-Val-L-[1-^{13}C]Pro-OCH_3. The pentapeptide methyl ester was saponified and converted to the *p*-nitrophenyl ester (-ONp) with bis(p-nitrophenyl) carbonate (Wieland et al., 1962), and the N^{α}-Boc group was removed with trifluoroacetic acid (TFA). A 0.9 M solution of the TFA salt of the peptide ONp in dimethyl sulfoxide was treated with 1.6 equiv of *N*-methylmorpholine and polymerized for 14 days. The reaction mixture was diluted with water with the formation of a sticky elastic mass, which went into solution after being stirred several hours in the cold room. The polymeric solution was dialyzed against a M_r 3500 cut-off dialysis membrane for 7 days and lyophilized.

[1-^{13}C]Gly^5-PPP. Boc-L-Val-L-Pro-OBzl (Prasad et al., 1985) was deblocked and coupled with Boc-[1-^{13}C]Gly-OH (90% enriched [1-^{13}C]glycine was obtained from MSD Isotopes, Rahway, NJ) by use of EDCI and HOBt to obtain Boc-[1-^{13}C]Gly-L-Val-L-Pro-OBzl. The synthesis was continued similarly by coupling one amino acid at a time to obtain Boc-Gly-L-Val-[1-^{13}C]Gly-L-Val-L-Pro-OBzl, which was hydrogenated, converted to *p*-nitrophenyl ester, and polymerized after the N^{α}-Boc group was removed as described above.

The peptide intermediates and the final polymers were characterized by comparing their NMR spectra and other physical properties with the materials synthesized in this laboratory as previously described (Urry & Prasad, 1985). In particular, in Figure 1A,B are the 25-MHz carbon-13 NMR spectra of L-[1-^{13}C]Pro^2-PPP and [1-^{13}C]Gly^5-PPP, respectively, in dimethyl sulfoxide, and in Figure 1C is the corresponding spectrum for the nonenriched polypentapeptide where all resonances are assigned (Urry et al., 1975). In Figure 1A the intense 99% carbon-13 enriched L-Pro^2 carbonyl carbon resonance is observed at 171.8 ppm (off scale), and the resulting $^1J(C'–C^{\alpha})$ splitting of the Pro^2 α-carbon is centered at 59.5 ppm. In Figure 1B is seen the intense 90% enriched carbonyl carbon resonance of Gly^5 at 168.5 ppm (also off scale). The α-carbon splitting for [1-^{13}C]Gly^5 is more difficult to demonstrate as this resonance comes under the dimethyl sulfoxide solvent peak at 41.6 ppm. These spectra serve to demonstrate the synthesis and purity of the singly carbonyl carbon enriched polypentapeptides of the present study.

Samples were prepared for the NMR experiments in 5-mm tubes by dissolving 0.3 g of lyophilized material in 1.5 mL of distilled H_2O. As deuterium exchange of the amide proton affects the relaxation of the carbonyl carbon in the peptide bond (Giannini et al., 1975), ^2H_2O was not used as a solvent

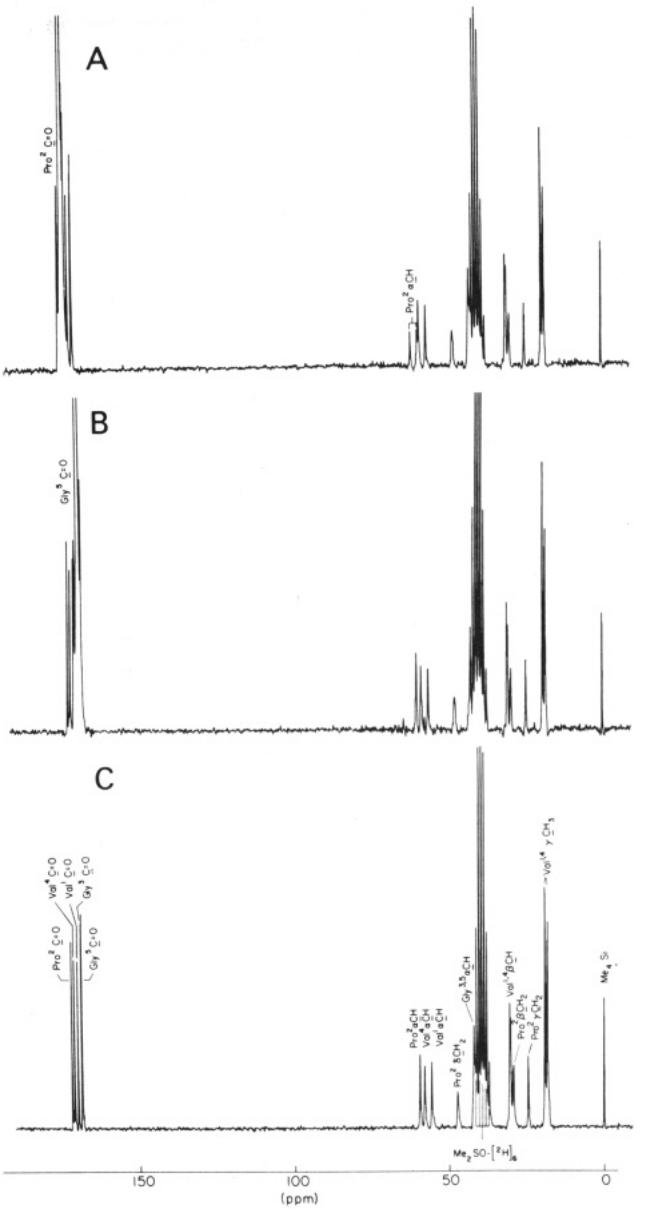

FIGURE 1: Carbon-13 NMR spectra at 25 MHz for the polypentapeptide of elastin in dimethyl sulfoxide at 30 °C: (A) 99% enriched L-[1-^{13}C]Pro^2-PPP; (B) 90% enriched [1-^{13}C]Gly^5-PPP; (C) nonenriched polypentapeptide with all resonances assigned. In (A), in addition to the intense Pro^2 carbonyl carbon resonance at 171.8 ppm, the Pro^2 α-carbon is seen to be split by $^1J(C'–C^{\alpha})$ coupling, and a peak on the upfield side of the enriched carbonyl carbon at 171.3 ppm is due to less than 10% of a cis Val–Pro bond. In (B), the enriched Gly^5 carbonyl carbon resonance is at 168.5 ppm with a peak at 168.1 ppm due to the cis Val–Pro bond of the repeating pentamer. The Gly^5 α-carbon splitting occurs under the solvent peak. In (C), the minor peaks due to the cis Val–Pro bond are just discernible. The absence of extraneous peaks and the occurrence of all the expected resonances verify the syntheses.

in these studies. The tubes were placed in a bath at 20 °C, and argon gas was passed through the samples for 6 h by means of long-tipped pipets placed into the bottom of the tubes. The tubes were capped under argon atmosphere and incubated at 30 °C in a bath for 3 days, during which time the polypentapeptide had formed a coacervate in the lower 4 cm of each tube. The equilibrium solution above the coacervates was removed at 30 °C, and the tubes were purged with argon and recapped. The result is referred to as the polypentapeptide coacervate concentration. NMR measurements were performed in order of increasing temperature (beginning at 10 °C and ending at 70 °C) so that the coacervates would not

be allowed to equilibrate at higher temperatures before a lower temperature measurement could be made. The samples were kept below coacervation temperature (20–22 °C) when measurements were not being performed.

The carbon-13 spin–lattice relaxation times (T_1's) and line widths were collected on a JEOL FX-100 nuclear magnetic resonance spectrometer equipped with a 5-mm ^{13}C–^{1}H dual probe operating at 25 and 100 MHz for these nuclei, respectively. This probe could be frequency locked by using either an internal or an external deuterium source. Probe temperatures were regulated to ±2 °C by a JEOL VT-3B variable-temperature controller and were measured directly in the NMR sampling area with a home-built temperature probe placed into a 5-mm tube containing water. Temperature measurements were made immediately before and after data collection at each temperature. Temperatures below 30 °C were attained by flowing cold nitrogen gas through a blast pipe from a 30-L Dewar flask containing liquid N_2 and then by warming the probe to the desired temperature.

The procedure for data collection at each temperature was carried out as follows: Temperatures were equilibrated with the proton irradiation on until stability was maintained for 0.5 h. Internal-lock (equivalent to sample) homogeneities were then adjusted on a sample of 20% acetaldehyde in [$^2H_6$]-benzene until a line width ≤ 0.25 Hz could be repeatedly measured on the –C*H*O signal. The lock was then switched to the external source, the first coacervate sample was placed in the probe, and then only spin homogeneities were adjusted by optimizing the proton free-induction decay from the H_2O signal of the sample. This tuning procedure had the added advantage of allowing the operator to select very accurately the frequency needed to center the proton broad-band irradiation. It should be noted that this frequency did shift to higher values as the temperature was increased. The probe was then switched to observe ^{13}C, and the 180° pulse width was determined. The above tuning and pulse-width adjustments required from 0.5 to 1 h to complete, allowing adequate time for temperature equilibration before the relaxation time measurement was carried out. As the enriched carbonyl carbon gave a strong NMR signal, only 8 or 16 pulse repetitions were required to accumulate adequate spectra. The second coacervate sample was similarly treated, and the probe temperature was redetermined after both measurements had been completed.

The inversion recovery method (180°–pulse interval–90°) was used to measure the relaxation times using from seven to nine partially relaxed spectra accumulated at various pulse intervals along with one fully relaxed spectrum having a pulse interval of greater than 5 times the enriched carbonyl carbon T_1. Rotational correlation times, τ_c, were calculated from the experimental T_1's with (Solomon, 1955; Allerhand et al., 1971)

$$\frac{1}{T_1} = \frac{\hbar^2\gamma_H^2\gamma_C^2}{10}\sum_j r_j^{-6}\left(\frac{\tau_c}{1+(\omega_H-\omega_C)^2\tau_c^2} + \frac{3\tau_c}{1+\omega_C^2\tau_c^2} + \frac{6\tau_c}{1+(\omega_H+\omega_C)^2\tau_c^2}\right) \quad (1)$$

which is the expression for the dipole–dipole (carbon–hydrogen) relaxation mechanism for an isotropically reorienting molecule. Here, γ_H and γ_C are the magnetogyric ratios for hydrogen and carbon-13, respectively, r_j is the internuclear distance from the carbonyl carbon to hydrogen j, and ω_H and ω_C are the resonance frequencies for hydrogen and carbon-13. The carbonyl line widths ($\nu_{1/2}$) were measured at half peak height from the spectrum of the fully relaxed signal (pulse interval ≥ $5T_1$) of the T_1 study. Assuming that $(\pi\nu_{1/2})^{-1} \approx T_2$, rotational correlation times were calculated with (Solomon, 1955; Allerhand et al., 1971)

$$\frac{1}{T_2} = \frac{\hbar^2\gamma_H^2\gamma_C^2}{20}\sum_j r_j^{-6}\left(\frac{\tau_c}{1+(\omega_H-\omega_C)^2\tau_c^2} + \frac{3\tau_c}{1+\omega_C^2\tau_c^2} + \frac{6\tau_c}{1+(\omega_H+\omega_C)^2\tau_c^2} + \frac{6\tau_c}{1+\omega_H^2\tau_c^2} + 4\tau_c\right) \quad (2)$$

which also assumes the same conditions for the transverse relaxation as for the longitudinal relaxation case above. Distances, r_j, for each carbonyl carbon in the pentamer sequence, L-Val^1-L-Pro^2-Gly^3-L-Val^4-Gly^5, were calculated from the crystal structure of the cyclic pentadecapeptide (Cook et al., 1980), a conformational correlate of the linear polymer that exhibits both carbon-13 and proton magnetic resonance properties in solution that imitate those of the polypentapeptide (Urry et al., 1981). As the dipole–dipole interaction decreases by the inverse sixth power, only those hydrogens that are within a 3-Å radius of the carbonyl carbon of interest were considered in the calculation of τ_c.

Results

As shown in Figure 2, the carbonyl carbon resonances for the PPP at the relevant viscous coacervate concentration are broad and overlapping. Because of this, efforts to determine motional properties from T_1 and line width are compromised and tend to lose the detail required to observe a relatively abrupt transition. The limitation is overcome by enriching only a single carbonyl carbon of the pentamer to levels at which the natural abundance carbons are negligible. The two chosen enrichments were the carbonyl carbon of the L-Pro^2 residue and the carbonyl carbon of the Gly^5 residue. The carbonyl carbon resonance for 99% L-[1-^{13}C]Pro^2-PPP at coacervate concentration is shown as a function of temperature in Figure 3. With this enrichment the T_1 and line-width values can be meaningfully determined. The spectra show a small upfield peak (<10%), which is due to the presence of some cis peptide linkage involving the Val–Pro bond. This minor peak becomes less apparent as the temperature is raised. The data for [1-^{13}C]Gly^5-PPP are similar.

In Figure 4 are plotted as a function of temperature the values of the longitudinal relaxation time (T_1) and $(\pi\nu_{1/2})^{-1}$, where $\nu_{1/2}$ stands for the full resonance line width at half-intensity. The quantity $(\pi\nu_{1/2})^{-1}$ is considered to be only an approximation to the transverse relaxation time, T_2, not due to field inhomogeneity (which can add no more than 0.25 Hz to the 4–12-Hz line widths) but rather due to the possibility of chemical shift heterogeneity occurring particularly in the 20–40 °C range, which could arise from different pentamer conformations interchanging slowly on the NMR time scale. Observed in Figure 4 is an abrupt transition beginning at about 25 °C and ending about 40 °C. This is a transition occurring in a viscoelastic medium in which the viscosity is between 10^3 and 10^4 cP.

As outlined under Materials and Methods, rotational correlation times were calculated from the data in Figure 4 with eq 1 and 2. The relationship between τ_c and T_1 and T_2 are plotted in Figure 5 for the summations of the carbonyl carbon–proton distances, i.e., $\sum_j r_j^{-6}$, relevant to the pentamer in the conformation found in the crystalline state of the cyclopentadecapeptide (Cook et al., 1980), which has been shown to be the cyclic conformatinal correlate of the linear polypentapeptide considered here (Urry et al., 1981).

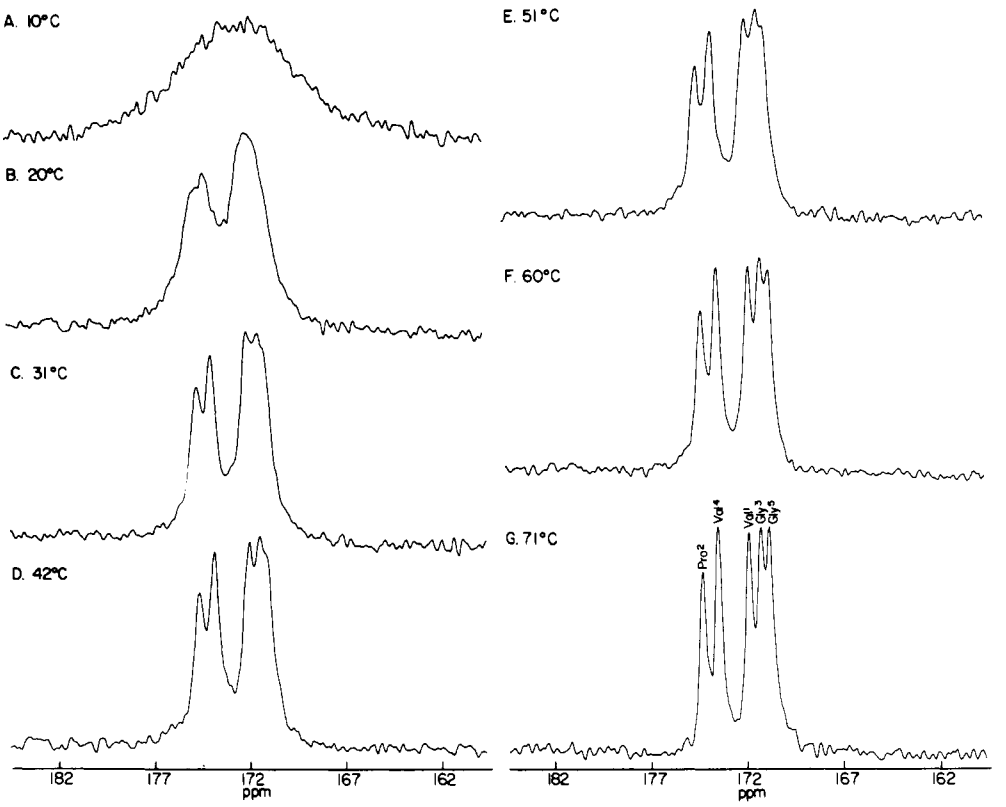

FIGURE 2: Carbon-13 NMR spectra in the carbonyl region of nonenriched polypentapeptide at coacervate concentration as a function of temperature. Efforts to use T_1 and line-width values derived from these spectra to characterize carbonyl mobility will lead to an obscuring of behavior due to overlapping of the five carbonyl carbon resonances.

It is apparent in Figure 5A that there are two possible values of τ_c for each experimental determination of T_1 whereas in Figure 5B there is one calculated value of τ_c for each experimental estimate of T_2. For this reason, the calculated values of τ_c derived from $(\pi\nu_{1/2})^{-1}$ will be considered first. The plot of log τ_c, where τ_c is derived from $(\pi\nu_{1/2})^{-1}$, vs. inverse temperature in kelvin is given in Figure 6B. What is observed for both the carbonyl carbons of the Pro^2 and Gly^5 residues is a decrease in magnitude of τ_c as the temperature is raised to 25 °C; between 25 and 40 °C, however, τ_c abruptly increases, and above 40 °C the rotational correlation time again decreases in magnitude as the temperature is raised to 70 °C. Since shorter correlation times mean greater mobility, the mobilities of the Pro^2 and Gly^5 carbonyl moieties increase up to 25 °C, they decrease on going from 25 to 40 °C, and then the mobilities resume their increase as the temperature increases above 40 °C. This result defines an inverse temperature transition and makes apparent the choice of the solution of τ_c derived from T_1. The plot of log τ_c derived from T_1 is plotted in Figure 6A, where the transition, uncomplicated by possible chemical shift heterogeneity, is more narrowly defined. The transition, as defined by the Gly^5 carbonyl carbon T_1 values, occurs within a 10 °C temperature range.

The slope of log τ_c vs. T^{-1} for the Pro^2 carbonyl carbon above 40 °C is well-defined and allows estimate of an energy of activation for the carbonyl mobility of 1.2 kcal/mol. The 40–70 °C slope is similar for the Gly^5 carbonyl in Figure 6A and for both carbonyls in Figure 6B. In spite of a sharp transition in the state of the polypentapeptide coacervate to a state of lower mobility, the energy barrier for the motion after the transition is very low.

Discussion

The polypentapeptide of elastin, (L-Val^1-L-Pro^2-Gly^3-L-Val^4-Gly^5)$_n$, is the most striking primary structural feature of tropoelastin (Sandberg et al., 1981), the precursor protein of fibrous elastin. The n of PPP is essentially 11 for pig and 13 for chick tropoelastins (L. B. Sandberg, private communication). That the sequence is found in species as evolutionarily removed as birds and mammals indicates that the PPP has been retained since the Mesozoic era through 100 million years of evolution. For such a sequence to be retained in separate lines of evolution over such an expanse of time one naturally concludes that it must provide a critical role in the function of fibrous elastin. The smallest available variation within each pentamer would seem to be unacceptable.

The PPP has properties very similar to those of tropoelastin and to α-elastin, a chemical fragmentation product of fibrous elastin; the latter is defined as the purified elastic component of connective tissue. All three exhibit the uncommon property of coacervation described in the introduction. The three coacervates have similar water content. When the small aggregates, which subsequently settle to form the coacervate, are themselves examined in the electron microscope after negative staining, similar filamentous structures are observed for each (Urry, 1982). Similar filamentous structures are observed for fibrous elastin itself (Gotte et al., 1976). When the PPP is chemically or γ-irradiation cross-linked, it is capable of an elastic modulus similar to that of fibrous elastin (Urry et al., 1976; Urry, 1984), and γ-irradiation cross-linked PPP (Urry et al., 1984) and fibrous elastin (Dorrington & McCrum, 1977) show similar thermoelasticity curves with a steep rise in elastomeric force on raising the temperature from 20 to 40 °C and with a near zero slope above 60 °C when log (force/temperature) is plotted vs. temperature (Dorrington & McCrum, 1977; Andrady & Mark, 1980). The latter suggests a large entropy component of the elastomeric force (Hoeve & Flory, 1974). The great advantage of studying the PPP is that it provides a simpler system with which to determine the mechanism of elasticity, and the simpler system

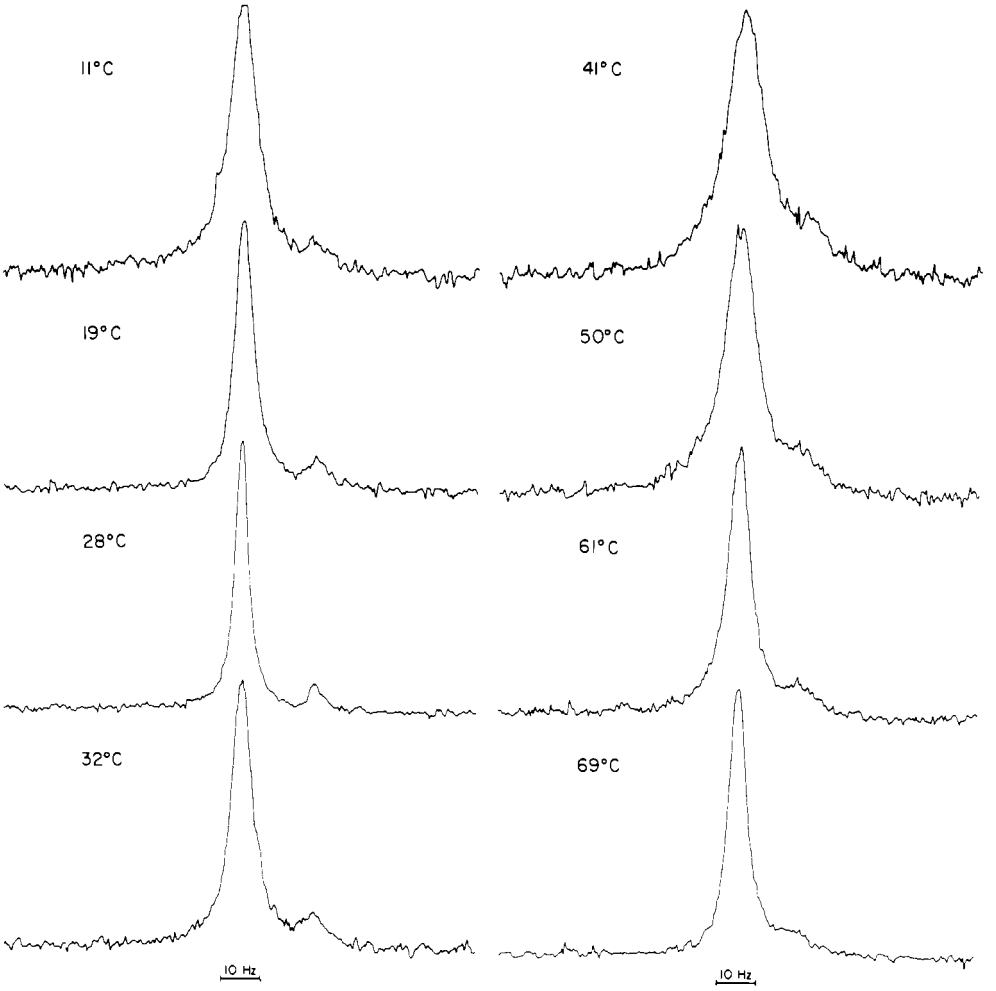

FIGURE 3: Temperature dependence of line shape of the Pro^2 carbonyl carbon resonance at the coacervate concentration of L-[1-^{13}C]Pro^2-PPP. The small peak at high field is due to a small amount of cis Val–Pro bond, which becomes less apparent above 40 °C.

is demonstrably very similar to the natural fiber.

The data of Figures 4 and 6 clearly demonstrate an inverse temperature transition. This abrupt transition implies that the 40 °C state is a more ordered state than the 20 °C state. The temperature-elicited aggregation, i.e., coacervation that coincides with the observed inverse temperature transition, was directly observed to be an aggregated state of increased intermolecular order by electron microscopy. Thus, the electron microscopic characterization of aggregation is an observation of increased order with increased temperature, and the inverse temperature transition is implied. On the other hand, the NMR relaxation studies directly observe the inverse temperature transition, and the increase in order is implied. The two separate observations are mutually corraborative. One necessarily concludes that the 40 °C (coacervate) state is a nonrandom state.

The coacervate state has also been demonstrated by circular dichroism to be a nonrandom state. Whereas the circular dichroism (CD) pattern of the polypentapeptide in water at 20 °C is characteristic of a polypeptide with limited order, the CD pattern of the coacervate is characteristic of a polypeptide containing a repeating β-turn (Urry, 1982; Woody, 1974), i.e., a repeating 10-atom hydrogen-bonded ring (Venkatachalam, 1968). By use of very high molecular weight PPP and very dilute concentrations, the process of coacervation has been followed stepwise from 15 to 70 °C, and there is observed a transition in the 20–40 °C temperature range that coincides with the transition reported here (Urry et al., 1985b). Furthermore, comparison of the conformations of cyclic analogues of the pentapeptide with n = 1–6 using proton and carbon-13 NMR have shown the cyclopentadecapeptide to have essentially the same conformation as the linear polypentapeptide and to exhibit nearly identical responses to temperature and to solvent (Urry et al., 1981). The crystal structure of cyclo-(L-Val^1-L-Pro^2-Gly^3-L-Val^4-Gly^5)$_3$ shows the type II Pro^2–Gly^3 β-turn (Cook et al., 1980) that had previously been demonstrated in solution, again with proton and carbon-13 nuclear magnetic resonance (Urry & Long, 1976; Urry, 1982). Thus, there is also an increase in intramolecular order with increase in temperature.

Another approach of characterizing carbonyl correlation times and of simultaneously obtaining information on polypentapeptide order is by means of dielectric relaxation studies. The planar peptide moiety, of which the carbonyl is the dominant dipolar component, has a large dipole moment, and the response of the dipole moment of peptide moieties within the coacervate to external fields, alternating at high frequencies, provides information on correlation times relevant to those measured here. Dielectric relaxation studies on the coacervate concentration of PPP in water, in the 1–1000-MHz frequency range, have demonstrated at 40 °C an intense, localized relaxation with a correlation time of 7 ns (Henze & Urry, 1985; Urry, 1984). The relaxation is not present at 20 °C but develops as the temperature is raised to 40 °C. On fitting the 40 °C curve with a Cole–Cole function (Cole & Cole, 1941; Grant et al., 1978), the correlation time distribution parameter, α, which allows for a range of correlation times, is essentially zero. Another means of demonstrating

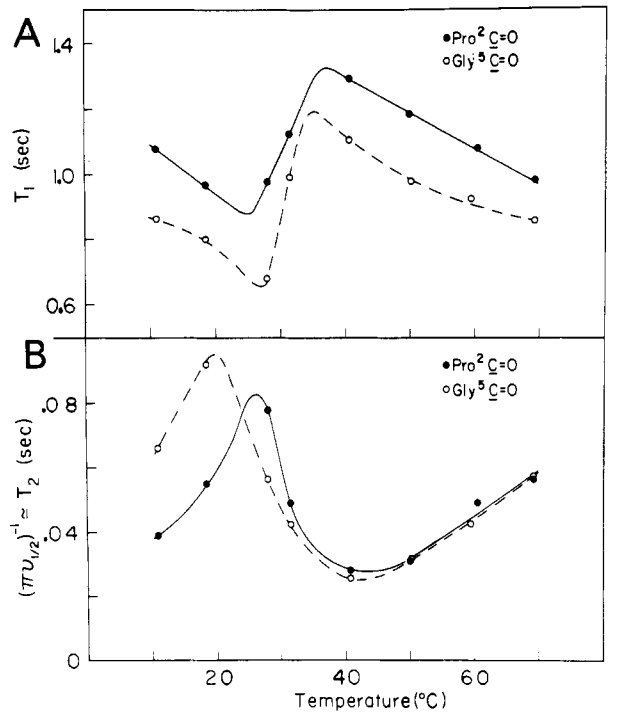

FIGURE 4: (A) Temperature dependences of the longitudinal relaxation time of the Pro² and Gly³ carbonyl carbon resonances of L-[1-^{13}C]-Pro²-PPP and [1-^{13}C]Gly⁵-PPP where a transition is observed in the 25–37 °C temperature range. (B) Temperature dependence of the Pro² and Gly⁵ carbonyl carbon resonance line widths of L-[1-^{13}C]-Pro²-PPP and [1-^{13}C]Gly⁵-PPP at their coacervate concentrations. As in part A, a transition is observed, but it appears to be somewhat broader, possibly due to chemical shift heterogeneities arising from repeating pentamers in different conformations.

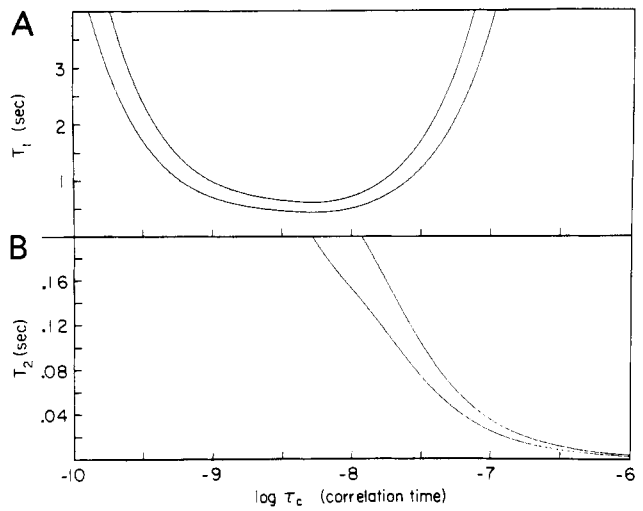

FIGURE 5: (A) Plot of eq 1 (T_1 vs. τ_c) for Pro² with $\sum_j r_j^{-6} = 3.83 \times 10^{46}/\text{cm}^6$ and for Gly⁵ with $\sum_j r_j^{-6} = 5.37 \times 10^{46}/\text{cm}^6$. (B) Plot of eq 2 ($T_2$ vs. τ_c) for Pro² and Gly⁵ of the polypentapeptide of elastin with the same carbonyl carbon–proton distances as in (A).

the spectrally localized nature of the relaxation is to fit the 20–40 °C difference curve with a Debye function where there is only a single correlation time. The fit is strikingly good (Henze & Urry, 1985). The simple Debye-type relaxation has been assigned to the rocking motion of peptide moieties, i.e., to a peptide librational process (Urry, 1984). The intense, localized nature of the transition argues that, during the transition to the coacervate state at 40 °C, the pentamers convert to a common conformation. This is because peptide librations in pentamers in different conformations would necessarily exhibit different correlation times. Since in a

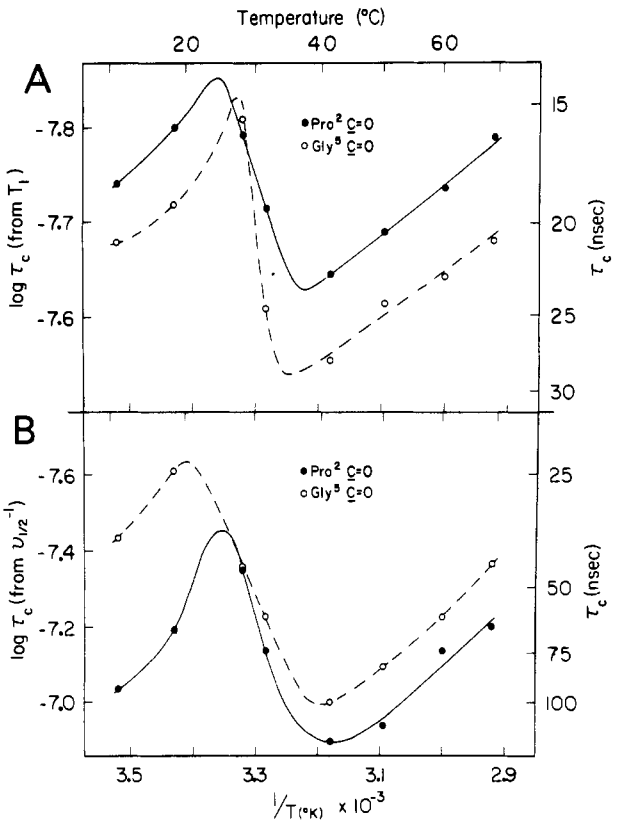

FIGURE 6: Plot of log τ_c vs. inverse temperature in kelvin: (A) τ_c derived from T_1 by means of eq 1; (B) τ_c derived from T_2 by means of eq 2. The correct solution for τ_c from T_1 was determined by first plotting (B) and then choosing the solution of eq 1 that gave the same relative changes in mobility on raising the temperature. In both cases, a transition to decreased mobility is observed in the 20–40 °C temperature range whereas mobility increases with temperature before and after the transition. The Gly⁵ curve in (A) defines a narrow transition occurring within a 10 °C change in temperature.

random conformation a broad range of correlation times would be observed (Lyerla & Torchia, 1975) as has been argued for rubber-like polymers (Schaefer, 1973) and as is reasonably the case for the polypentapeptide at temperatures below 25 °C [see Figure 1 of Henze & Urry (1985)], the observation of a single, intense localized relaxation at 40 °C indicates a regular nonrandom conformation. Thus, dielectric relaxation studies also argue for increased intramolecular order for the transition characterized here as an inverse temperature transition.

A determination of the correlation time from dielectric relaxation studies does not require an involved theory but rather depends on the fitting of a relaxation spectrum to a relatively simple expression. In this regard, it is not subject to the uncertainties of the simplifying assumptions used to calculate correlation times from NMR relaxation times. When the dielectric relaxation is of the Debye-type (Grant et al., 1978; Frohlich, 1958), as it is for the 7-ns relaxation of the polypentapeptide coacervate (Henze & Urry, 1985; Urry, 1984), determination of correlation times is unambiguous, and the value can be usefully compared to those derived from the T_1 and line-width studies. Interestingly, the correlation time observed by dielectric relaxation is such that, with the 25-MHz observation frequency for carbon-13, the product $\omega\tau_c$ would equal 1, and T_1 is, of course, expected to differ from T_2 [$=1/(\pi\nu_{1/2})$]. On the basis of the dipole–dipole relaxation mechanism as represented by eq 1 and 2 above, when $\omega\tau_c = 1$, $T_1 = 2.4T_2$. Experimentally, $T_1 \approx 45T_2$ at 40 °C. While it is not the purpose of this paper to analyze relaxation

mechanisms, it should be noted that the dielectric relaxation results make the polypentapeptide coacervate a system of particular interest for considering carbonyl carbon relaxation mechanisms in viscous media ($\eta > 10^3$ cP).

Given the discrepancy between the experimental T_1/T_2 ratio and the calculated value based on the assumption of isotropic motion and dipole–dipole relaxation mechanism, it is perhaps surprising that at 40 °C the magnitude of the correlation times derived by the dielectric relaxation and NMR longitudinal relaxation methods differs by only a factor of 3 (see Figure 5A). The discrepancy with the line-width-derived correlation times is more nearly the same as the discrepancy in calculated and experimental T_1/T_2 ratios. This is the case especially at 40 °C after the conformational transition is essentially complete and where $\tau_c(T_2)$ is greater than 100 ns (see Figure 5B). Lyerla & Torchia (1975) have made the important point that the NMR-derived correlation time is an average quantity. Even though the dielectric relaxation results indicate at 40 °C that there is a discrete relaxation process at 7 ns (Henze & Urry, 1985; Urry, 1984) rather than a wide range of correlation times near 7 ns, there is the suggestion that an additional relaxation occurs at longer correlation times near several hundred nanoseconds. Accordingly, at 40 °C the proper averaging process would appear to be one that considered discrete but widely displaced correlation times. In such a case, the NMR values would be expected to be longer than the single dielectric relaxation characterized in the 1–1000-MHz frequency range.

In spite of the limitations in obtaining complete understanding of the T_1 and T_2-derived correlation times, it is of particular interest to consider the magnitude and temperature dependence of τ_c once the transition has been completed. A sharp transition has been clearly demonstrated to a state of increased order at 37 °C where the calculated correlation times in the viscous coacervate are as short as 25 ns. This requires, of course, that a 25-ns correlation time and even a calculated 16-ns τ_c at 70 °C cannot incautiously be used to argue for the absence of order. With respect to the temperature dependence, all four curves for the plot of log τ_c vs. T^{-1} in the 40–70 °C range show relatively constant and similar slopes. From the slopes, one can calculate an experimental activation energy of 1.2 kcal/mol, which is likely an underestimate of the barrier as secondary relaxation mechanisms such as spin–rotation may have decreased the slope. This low barrier for motion occurs in spite of the evidence for the formation of a regular, nonrandom structure, and it corresponds reasonably well with the value of 1.6 kcal/mol obtained by Aaron & Gosline (1981) from the stress-optical coefficient of single fibers of ligamentum nuchae. The thermodynamics of the regular structure resulting from the inverse temperature transition is such as to be compatible with the elasticity of biological elastic fibers. Finally, it may be noted that a regular conformation of the polypentapeptide, called a β-spiral, has been described (Venkatachalam & Urry, 1981) that exhibits peptide liberational processes with the required low activation energies (Urry et al., 1982; Urry, 1984).

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Registry No. PPP, 69289-41-4; PPP, SRU, 69221-84-7; [1-^{13}C]Pro^2-PPP, 97352-66-4; [1-^{13}C]Pro^2-PPP, SRU, 97352-69-7; [1-^{13}C]Gly^5-PPP, 97352-68-6; [1-^{13}C]Gly^5-PPP, SRU, 97352-70-0; [1-^{13}C]Pro-OMe, 97352-63-1; BOC-L-Val-OH, 13734-41-3; BOC-Gly-L-Val-Gly-OH, 96847-92-6; BOC-L-Val-L-Pro-OBzl, 58872-03-0; BOC[1-^{13}C]Gly-OH, 97352-64-2.

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Proton NMR Spectroscopy of Sulfmyoglobin†

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ABSTRACT: The ^1H NMR spectra of ferrous sulfmyoglobin, metsulfmyoglobin, and ferric cyanosulfmyoglobin were obtained at 300 MHz. Hyperfine-shifted resonances are observed in the case of metsulfmyoglobin and ferric cyanosulfmyoglobin that have line widths and cover a chemical shift range that are comparable to the corresponding forms of normal myoglobin. Two methyl resonances are observed in the spectrum of ferric cyanosulfmyoglobin at 44.19 and 25.48 ppm (25 °C, pH* 8.3) that have been assigned to heme methyls at the 8- and 5-positions on the basis of pH titration effects homologous to the corresponding methyl resonances in ferric cyanomyoglobin. Examination of aromatic region resonances and the pH titration profiles of histidine resonances lead to the conclusion that the overall conformation of sulfmyoglobin was highly homologous to that of normal myoglobin and afforded assignments of histidine residues of the former. The most likely position for the addition of a sulfur atom to the heme of sulfmyoglobin is pyrrole ring A, with ring B a possible, but less likely, alternative.

Sulfmyoglobin[1] and sulfhemoglobin are unusual derivatives formed from the respective normal globins by the addition of sulfur to the porphyrin skeleton of the prosthetic group heme. This addition leads to conversion of the globins to bright green proteins with characteristic absorption bands in their optical spectra above 600 nm. Although the formation of a sulfglobin was first observed in the 1860s, the precise structure of the modification has never been established.

The existence of sulfglobins is more than a laboratory curiosity. SHb has been reported to be formed in vivo under certain pathological conditions or in the presence of high dosages of some drugs related to common analgesics such as phenacetin [Morell et al. (1967) and references cited therein]. Increased levels of SHb have been correlated with exposure to chemical pollutants (Bucley, 1982; Lambert et al., 1982; Mills et al., 1982). The clinical determination of sulfglobins has remained a major concern (Drabkin & Austin, 1935; Zwart et al., 1984; Tomada et al., 1983).

Several groups have made major contributions to the available structural knowledge of sulfglobins (Michel, 1938a,b; Nicholls, 1961; Morell et al., 1967; Berzofsky et al., 1971a, 1972b) that will be briefly summarized as follows. The unusual green color is not a consequence of the coordination geometry of the central iron but, instead, is due to covalent modification of the porphyrin skeleton. The modification is most probably the reduction of the skeleton to the level of a chlorin. The best available evidence indicates that a single sulfur atom is incorporated, most likely as an episulfide. The in vitro formation of sulfglobins requires the reaction of the Fe(IV) oxidation state with stoichiometric amounts of HS^-. A mechanism has been proposed to account for the in vivo production of sulfglobins on the basis of chemical species known to be present in erythrocytes (Nichol et al., 1968). The sulfglobins are somewhat unstable and can readily decompose. In this process they revert back to apparently unmodified myoglobin. Sulfheme can be extracted from the globin but has a short lifetime, quickly reverting to normal protoheme (Berzofsky et al., 1972b).

The physiochemical properties of the intact green globins have been systematically investigated. The optical spectra of a variety of forms have been reported (Berzofsky et al., 1971a,b, 1972a,b; Nicholls, 1961; Carrico et al., 1978). The infrared (Berzofsky et al., 1972a,b; Carrico et al., 1978) and magnetic circular dichroism (Brittain et al., 1982) spectra have been investigated. Magnetic properties have been charac-

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[1] Heme nomenclature and abbreviations are as follows: The structure of the heme prosthetic group of hemoglobins and myoglobins is shown in Figure 1 with the substituent positions labeled according to a conventional scheme. This heme is commonly referred to as protoheme, heme *b*, or iron protoporphyrin IX. The organic macrocycle is a porphyrin when fully aromatic and is called a chlorin when one of the pyrrole rings has been saturated at the β carbons to acquire two extra substituents. The β carbons are the outermost of the pyrrole rings and bear the substituents. An episulfide is the sulfur analogue of an epoxide. In the proposed substructure for the sulfglobins, the covalently added sulfur bridges two adjacent β-pyrrolic carbon atoms forming a three-atom ring. The term "meso" refers to the methine bridge carbons linking the four pyrroles. The term heme will be used in this paper to denote the iron porphyrin complex without regard to oxidation state of the iron or ligands at the fifth and sixth axial coordination sites. The term sulfheme will be used to refer to the product of covalent addition of the sulfur moiety. The abbreviations Hb and Mb will respectively represent hemoglobin and myoglobin, and SHb and SMb will represent the corresponding sulfur-modified forms. When so used, the context will be general, and nothing is implied about the oxidation or ligation states. When necessary to specify these, the following abbreviations will be used: $SMbFe^{III}$, the oxidized or met form; $SMbFe^{II}$, the ferrous deoxy form; $SMBFe^{III\text{ or }II}X$, the given oxidation state with X as a ligand. For example, $SMbFe^{III}CN$ is the ferric cyano complex of sulfmyoglobin.

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