brief communication

Electron spin relaxation measurements on the blue-copper protein plastocyanin

Deviations from a power law temperature dependence

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ABSTRACT Electron spin relaxation rates over the temperature range 1.41–15.6 K are presented for the copper-containing protein plastocyanin. Measurements are described for two samples, each derived from a different preparation of equivalent purity, for which the ionic, redox, and protein compositions varied slightly. X-band data are analyzed in terms of a phonon-limited direct process and a Raman relaxation process, where the index of the Raman transport integral is treated as a fitting parameter. Both samples yield rate data at the highest temperatures that are characterized by small deviations from a simple T^n power law dependence, with n in the range 4.8–5.2. These deviations are most easily quantified when the T^n power law fits are compared with similar functions that allow for a finite cutoff in the phonon density of states corresponding to Debye temperatures between 90 and 100 K with n in the range 5.0–5.5.

INTRODUCTION

Electron spin-lattice relaxation rates from metalloproteins have been characterized by an anomalous T^n temperature dependence for the Raman rate (1–5). The Raman electron spin relaxation process is an inelastic, two-phonon scattering mechanism that balances the magnetic energy of a spin flip with the change in vibrational energy between two lattice modes. In the ordinary Debye approximation, the density of vibrational states is given by

$$\rho(\nu) \propto \nu^{m-1} \qquad 0 \le \nu \le \nu_{max}, \tag{1}$$

where m is the spectral dimension and $\rho(\nu) = 0$ for $\nu > \nu_{max}$. The Raman rate, $1/T_{1R}$, for an ion with an odd number of electrons (Kramers' ion) is then described by

$$1/T_{1R}(T) \propto \int_0^{\nu_{max}} \frac{(\nu^{m-1})^2 \nu^4 \exp(h\nu/kT)\, d\nu}{[\exp(h\nu/kT) - 1]^2}. \tag{2}$$

This can be rewritten in terms of a transport integral

$$1/T_{1R}(T) = CT^{3+2m} \int_0^{\Theta/T} \frac{x^{2+2m} \exp(x)\, dx}{[\exp(x) - 1]^2} = CT^{3+2m} J_{2+2m}(\Theta/T), \tag{3}$$

where $x = h\nu/kT$, $\nu_{max} = k\Theta/h$, and Θ is the Debye temperature. At temperatures sufficiently below Θ, the integral expression for the Raman relaxation rate reduces to a simple T^n power law,

$$1/T_{1R}(T) = CT^n J_{n-1}(\infty) = C_\infty^n T^n, \tag{4}$$

where $n = 3 + 2m$.

The simple form of Eq. 4 reflects the fact that the highest energy phonon states are not occupied and that the system is indistinguishable from one in which the ν_{max} of Eq. 1 is infinite. Theory predicts a spectral dimension of three and a T^9 Raman rate for Kramers' ions at low temperatures in crystals. Previous measurements on heme (1–5), iron-sulfur (4), and copper (5) proteins indicated that the temperature dependence of the relaxation rates could be fit to a strict T^n relationship. These results were consistent with a simple power law for $\rho(\nu)$ and a large value of Θ. The values of m, however, were between one and two. This anomalous temperature dependence led us to propose a fractal model of relaxation in proteins, based on the fractional value of the Raman exponent. Our collective measurements have since lead to considerable theoretical interest in the spin-lattice relaxation properties of biopolymers (6–15).

In this communication, we report evidence that a simple power law formalism for the Raman temperature dependence may be inadequate to account accurately for the highest temperature data of some blue-copper proteins. In contrast to earlier results from various iron proteins, preliminary data (16, 17) on azurin from *P. aeruginosa* are characterized at the highest temperatures by rates that are noticeably slower than those predicted by a T^n relationship. To understand this new facet of protein relaxation, we began a systematic study of two blue-copper proteins. Since copper proteins typically relax more slowly than low-spin iron proteins, we are able to study, within our current experimental limits, a considerably wider temperature range. This has the benefit of maximizing any deviations from a T^n dependence that might otherwise be unattainable in equivalent measure-

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ments of iron proteins. Our attention in this report concentrates on the blue-copper protein plastocyanin; a more comprehensive study of the related protein, azurin, is in preparation and will be published later. Preliminary accounts of the data from these proteins have been presented (18, 19).

EXPERIMENTAL PROCEDURES

Plastocyanin is a paramagnetic copper-containing protein comprised of a single polypeptide chain with a mass of 10,500 D and a single copper ion. X-ray crystallographic data at 1.6 Å resolution (20) reveal the single copper ion is buried 6 Å inside the protein matrix, within a hydrophobic environment, and bound to the protein through two nitrogen and two sulfur atoms in a near-tetrahedral symmetry.

Plastocyanin was extracted from spinach according to our previous modification (21) and then purified by standard procedures (22). Oxidized plastocyanin was purified to an A^{278}/A^{597} absorption index of 0.85 after the final Sephadex G-75 gel filtration chromatography. Two preparations of equivalent purity were used in this study. Electron paramagnetic resonance spectra of both preparations did not detect any other active species, including extraneous (type II) copper (21). Moreover, because initial observations indicated plastocyanin EPR spectra are not affected by a lyophilization–dissolution cycle, each preparation was lyophilized for transit from Italy to the USA.

Each lyophilized preparation of plastocyanin was used to make corresponding relaxation samples, I and II. Both samples for the relaxation measurements were prepared by dissolving the protein in aqueous potassium phosphate (pH 7.0), centrifuging, and concentrating by ultrafiltration to a final volume of 0.4 ml. The ionic strength of the phosphate buffer used in sample I and sample II was 0.43 and 0.085, respectively. The two samples also differed slightly in total protein concentration and the relative fraction of the Cu^{2+} species. Plastocyanin concentrations in relaxation samples were determined spectrophotometrically on an aliquot diluted with 50 mM KP_i (pH 7.0) and oxidized with a small excess of potassium ferricyanide; values are based on an absolute millimolar extinction of 4.9 at 597 nm (23). The amount of oxidized copper in a relaxation sample was also determined from optical absorption measurements at 597 nm on two portions of the same diluted aliquot. One portion was used to examine the reduced (ascorbate) form, while the second was used to probe the oxidized (ferricyanide) equivalent; both assays provided an estimate for the unmodified proportion of oxidized copper. These measurements indicated sample I contained 0.62 mM Cu^{2+} and 0.97 mM total copper, whereas sample II contained 0.18 mM Cu^{2+} and 0.54 mM total copper. Reasons for the partial reduction of plastocyanin in these samples are not apparent. The known instability of plastocyanin at this high level of purity (22–24) and the long period of storage (weeks to months) in the lyophilized state may have contributed to the partial reduction of copper in the relaxation samples. Our methods for preparing relaxation samples do not produce a similar effect with highly purified preparations of oxidized azurin.

The two plastocyanin solutions were each frozen in the bottom half of a rectangular TE_{101} copper cavity and stored in liquid nitrogen before relaxation measurements. The cavity is designed to maximize the magnetic filling factor and minimize dielectric losses. Electron spin-lattice relaxation rates are measured at X-band microwave frequencies using signal averaging and the pulse-saturation/recovery technique (4). The system is capable of measuring exponential recoveries as fast as 2×10^5 s^{-1}, as determined by measurements (unpublished) on a single crystal of neodymium-doped lanthanum magnesium nitrate $[La_2Mg_3(NO_3)_{12} \cdot 24H_2O{:}Nd]$. Signal-to-noise constraints limited the acquisition of protein data to rates $<5 \times 10^4$ s^{-1}. Recovery of the saturated signal to the thermal equilibrium value is monitored with a signal averager and stored on magnetic disk for analysis. Temperatures between 1.4 and 25 K are measured and controlled to within ±4 mK using the output from a bridge circuit that incorporates a calibrated germanium resistance thermometer, which is mounted directly to the upper half of the copper cavity. Through the use of pretrigger sampling we are able to determine the equilibrium baseline signal, independent of the dwell time of the signal averager.

Methods of data reduction are modified from our earlier work. Previous rate versus temperature data were analyzed using weighting factors that assumed a constant percentage error (i.e., weighted inversely as the square of the respective rate). This assumption is reasonable, provided that all rates have an equal relative deviation, which is typically ~ ±5%. As the rates approach or exceed 10^4 s^{-1}, this assumption is not always valid. Since our signal recovery profiles are closely approximated with an exponential time dependence, a properly weighted linear regression of the logarithm of the signal versus time provides a simple method for determining the experimental relaxation rate ($Rate_i$) and its associated uncertainty (σ_i). For subsequent fits of the rate versus temperature, a weighting factor equal to σ_i^{-2} is utilized.

Analyses of rate versus temperature data are performed using a nonlinear, weighted least-squares program and a generalized functional form

$$1/T_1(T) = AT/(1 + B/T) + CT^n J_{n-1}(\Theta/T), \quad (5)$$

which includes terms for both a direct and a Raman relaxation process. The first term in Eq. 5 represents a partially phonon-limited (bottlenecked) direct relaxation process and is only dominant at our lowest temperatures (25–30). Depending on the value of B, which is determined by the degree to which the system is phonon limited, the log-log slope of $1/T_1$ vs. T varies between one (no phonon bottleneck, $B \ll T$) and two (extreme phonon bottleneck, $B \gg T$). This form of the temperature dependence of a phonon bottleneck is an approximation that is valid under our operating conditions. The precise temperature dependence of an extreme phonon bottleneck [$\coth^2(h\nu/2kT)$], has been observed in relaxation data from myoglobin taken at 16 GHz and at temperatures below 1 K (31).

The second term in Eq. 5 includes the complete Raman integral expression of Eq. 3. Since numerical values of the transport integral, $J_{n-1}(\Theta/T)$, are published only for integer values of n (32), our nonlinear regression analysis incorporates a fast-converging algorithm to solve the transport integral in Eq. 5 for any iterated value of n and the Debye temperature. Previous fits to protein data fixed Θ at infinity and solved only for values of n.

Three statistical and dimensionless quantities are useful in evaluating the significance of the data and the analyses (33). The first, σ_r^2, is a weighted average of the individual relative variances, $(\sigma_i/\text{Rate}_i)^2$. We choose a simple, yet appropriate, weighting factor w_i for these relative variances, $w_i = (\sigma_i/\text{Rate}_i)^{-2}$, which yields for the first quantity

$$\sigma_r^2 = N/\sum_i (\text{Rate}_i/\sigma_i)^2. \quad (6)$$

σ_r^2 provides an overall estimate for the relative precision of a given data set and is independent of any fitting function. The second quantity, χ_r^2, is the reduced chi-squared from the weighted nonlinear analysis. χ_r^2 provides a measure of how well the functional form agrees with the data set. The third quantity, QOF, is the product of σ_r and χ_r. QOF provides an overall quality of fit criterion to compare different data sets.

RESULTS AND DISCUSSION

Relaxation data for two samples of plastocyanin are presented in Fig. 1. The agreement of each data set with the two theoretical models considered in this study, which correspond to a finite or an infinite Θ in Eq. 5, are further illustrated in Fig. 2. The best-fit parameters to these models, along with statistical data for the fitting functions, are summarized in Table 1.

In plastocyanin, as in other protein relaxation data (3), samples with slower relaxation rates at the lowest temperatures are associated with larger values of n in the Raman region. The analysis of sample II (in Fig. 2 and Table 1), however, shows clearly that a simple power law is inadequate to fit the relaxation data in the highest temperature region. Similar conclusions are less obvious for sample I; both models yield fits that lie above the highest tempera-

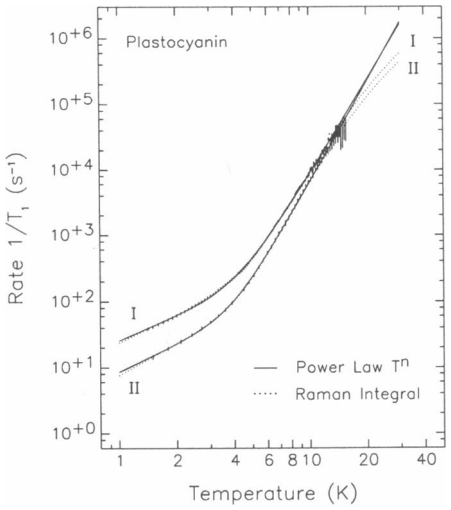

FIGURE 1 Cu^{2+} electron spin relaxation rates from two samples of plastocyanin. Sample I measurements, at 9.502 GHz and $g = 2.054$, range from 1.41 to 14.5 K in 0.1-K intervals (133 data points), while sample II measurements, at 9.497 GHz and $g = 2.053$, range from 1.5 to 15.6 K in 0.3-K intervals (47 data points). Shown for each sample are the data with their associated uncertainties (|) and the two best-fit functions, which correspond either to an infinite (power law T^n) or a finite upper-limit to the Raman integral (Eq. 5).

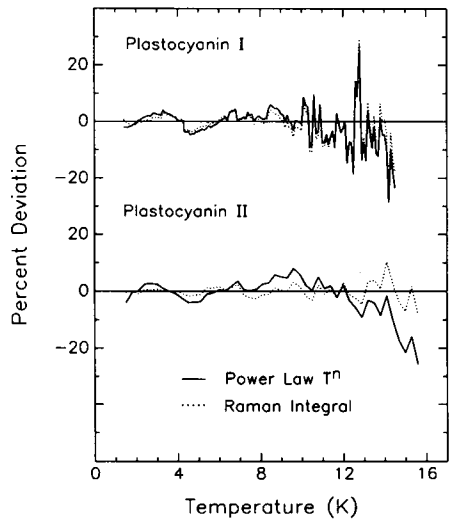

FIGURE 2 Point-by-point percentage deviation of the data from the two best-fit functions for both samples of plastocyanin.

TABLE 1 Relaxation parameters of plastocyanin at a magnetic field of 330.5 mT and microwave frequencies of 9.502 GHz (sample I) and 9.497 GHz (sample II)*

Sample	Direct process		Raman process				Statistics		
	A	B	n	C	C_{∞}^{n}	Θ	σ_r	χ_r^2	QOF
	$s^{-1}K^{-1}$	K		$s^{-1}K^{-n}$	$s^{-1}K^{-n}$	K			
I	34.8	0.38	4.81	6.55E-03	1.30E-01	∞	4.66	1.65	5.99
	(7.3)	(47)	(0.7)	(7.8)	(7.8)				
I	40.4	0.72	4.98	3.67E-03		99.7	4.66	1.58	5.84
	(10)	(37)	(1.7)	(30)		(8.4)			
II	14.2	0.66	5.14	1.41E-03	4.50E-02	∞	7.14	0.27	3.69
	(8.3)	(34)	(0.6)	(6.9)	(6.9)				
II	19.9	1.62	5.47	4.50E-04		89.5	7.14	0.07	1.83
	(6.4)	(14)	(0.9)	(17)		(2.7)			

*Values in parentheses correspond to percentage equivalents of the relative uncertainty for the parameters.

ture data and the statistical parameters in Table 1 are nearly identical. Using the F statistic as a significance test, a closer examination of the fits for sample I does reveal there is a <1% probability that the improvement achieved by the additional parameter (finite Θ) is purely random. This deviation from a simple power law dependence with plastocyanin samples represents a marked contrast to previous relaxation data on metalloproteins. Also significant from the viewpoint of relaxation models is the wide variation (4.81–5.47) in the observed values of n in samples that differ so little in preparation. In only one of the four analyses (sample II, finite Θ) do we obtain an n value that is close to the 5.64 ± 0.08 value calculated from estimates of the chain fractal dimension for popular plastocyanin (supplemental data to reference 4, using 1PCY).

Previous work on cytochrome C551 and putidaredoxin (4), as well as a reinvestigation of myoglobin azide (3), indicated n values were dependent on ionic and cosolvent conditions of the protein samples. The effect of higher ionic strength on cytochrome C551 and putidaredoxin was to increase the values of n in the relaxation data. The effect of ionic strength on n values for myoglobin azide was negligible, but the effect of a glycerol cosolvent was to produce a significant rise in the spectral dimension. Studies on myoglobin azide (3) indicate very high protein concentration also tends to increase the spectral dimension. In these cases, the maximal values of n approximated the chain fractal dimension calculated from the protein's x-ray coordinates. The results from this study underscore the ever increasing complexity in deriving physical significance for the spectral dimensions. Contrary to the results from the heme and iron-sulfur proteins cited above, plastocyanin data show a decrease in the spectral dimension with both a moderate increase in ionic strength and an increase in protein concentration.

Plastocyanin relaxation rates in the low temperature region also exhibit a dependence on the chemical composition of the samples. Rates attributable to the direct process are seen to increase with a moderate increase in either ionic strength and/or total protein/spin concentration. Unpublished azurin data show a similar qualitative behavior with plastocyanin; higher spin concentrations tend to yield faster relaxation rates in the lowest temperature region. Previous data on cytochrome C551, putidaredoxin, myoglobin azide (3, 4), and azurin (unpublished) indicate that higher ionic strength decreased the magnitude of the rates in the lowest temperature region.

Plastocyanin data in the lowest temperature region are adequately fit to a partially bottlenecked direct relaxation mechanism. Nonzero values of B are required to fit the lower temperature data because the log-log slope in this region is between one and two. Only the measurements on cytochrome C551 (4) have required a partially bottlenecked direct process. There are, however, some inconsistencies with this interpretation of the direct process for plastocyanin. In terms of only a spin concentration dependence, the ordinary model of a phonon-limited direct process would require A to remain constant and B to increase linearly with the concentration of the paramagnetic species. Plastocyanin A values are found to increase by a factor of 2.03 (fit with finite Θ) to 2.45 (fit with infinite Θ) with increasing concentration. Despite their limited precision, B values actually decrease by a factor of 0.44 (fit with finite Θ) to 0.58 (fit with infinite Θ) with increasing concentration. Samples I and II have a spin concentration ratio of 3.44 and a protein ratio of 1.8.

CONCLUSIONS

The present study indicates that Raman relaxation rates in metalloproteins are not universally described by a simple power law in temperature. This observation alone

poses a significant constraint on the physical interpretation of relaxation parameters. The need to include a finite Debye temperature in the analysis of the plastocyanin data does not improve our ability to interpret the corresponding spectral dimension. Although one value of n nearly agrees with our estimate using the chain fractal dimension for poplar plastocyanin, the other value of n from a sample of similar composition deviates significantly; the evidence is accumulating that the chain fractal dimension is not simply correlated with relaxation behavior in proteins. Because all available spectroscopic data indicate the samples retain the native conformation of plastocyanin, we are unable to conclude that these spectral dimensions correlate with dissimilar structures. The estimate of a Debye temperature near 100 K implies a length scale ($\lambda/2$) of 5 Å, which is consistent with the monomer (alpha carbon) spacing of ~4 Å in proteins. In fact most, if not all, of our previous measurements can be modeled with the complete expression given by Eq. 5. Our analysis yields Debye temperatures of similar magnitude, but the precision of these data has never justified the need to report an analysis that included an additional parameter. What is apparent from this study is the need for more high-quality data and more comprehensive analytical functions to model the relaxation rates. Whereas earlier measurements on iron proteins, with faster relaxation rates, may have masked our experimental window, the results of copper proteins, both here and unpublished, indicate numerous complexities are inherent in the analysis of protein data. At present, the anomalous temperature dependence of the Raman relaxation rates in proteins and their variation with ionic, solvent, and concentration conditions pose a significant challenge to the formulation of a satisfactory relaxation model.

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