Aggregation states of mitochondrial malate dehydrogenase

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Abstract

The oligomeric state of fluorescein-labeled mitochondrial malate dehydrogenase (L-malate NAD$^+$ oxidoreductase; mMDH; EC 1.1.1.37), as a function of protein concentration, has been examined using steady-state and dynamic polarization methodologies. A "global" rotational relaxation time of 103 ± 7 ns was found for micromolar concentrations of mMDH–fluorescein, which is consistent with the reported size and shape of mMDH. Dilution of the mMDH–fluorescein conjugates, prepared using a phosphate buffer protocol, to nanomolar concentrations had no significant effect on the rotational relaxation time of the adduct, indicating that the dimer–monomer dissociation constant for mMDH is below 10^{-9} M. In contrast to reports in the literature suggesting a pH-dependent dissociation of mMDH, the oligomeric state of this mMDH–fluorescein preparation remained unchanged between pH 5.0 and 8.0. Application of hydrostatic pressure up to 2.5 kilobars was ineffective in dissociating the mMDH dimer. However, the mMDH dimer was completely dissociated in 1.5 M guanidinium hydrochloride. Dilution of a mMDH–fluorescein conjugate, prepared using a Tris buffer protocol, did show dissociation, which can be attributed to aggregates present in these preparations. These results are considered in light of the disparities in the literature concerning the properties of the mMDH dimer–monomer equilibrium.

Keywords: dissociation; fluorescence polarization; mitochondrial malate dehydrogenase; time resolved

Porcine heart mitochondrial malate dehydrogenase (mMDH) [(S)-malate:NAD$^+$ oxidoreductase, E.C. 1.1.1.37] is a dimeric enzyme composed of identical subunits, each with 314 amino acids, which catalyzes the interconversion of L-malate and oxaloacetate utilizing NAD$^+$ as a coenzyme. In 1970s and 1980s, several groups studied the dimer–monomer equilibrium of mMDH using different approaches. Shore and Chakrabarti (1976) reported fluorescence polarization studies on enzyme labeled with either FITC or fluorescamine and, in both cases, reported finding a concentration-dependent dissociation with a dissociation constant (K_d) equal to 2 × 10^{-7} M at 23 °C in pH 8.0, 50 mM Tris-acetate buffer. Bleile et al. (1977) and Hodges et al. (1977) reported gel filtration chromatography and sedimentation velocity ultracentrifugation studies indicating dissociation constants for the dimer similar to those reported by Shore and Chakrabarti. Frieden et al. (1978), however, reported enzyme kinetic studies suggesting that mMDH did not undergo dissociation even at 10^{-9} M. Jaenicke et al. (1979), using gel filtration chromatography, also concluded that mMDH re-mained a dimer over the concentration range of 1.67 × 10^{-6} M to 2.9 × 10^{-9} M in 0.2 M phosphate buffer, pH 7.6, 20 °C.

Wood et al. (1978, 1981) and Hodges et al. (1977) reported a pH dependence of the dimer/monomer equilibrium, suggesting that pH values below 7 promote dissociation; specifically, a dissociation constant greater than 2 × 10^{-4} M was reported at pH 5.0, while at pH 7.5 this value was given as less than 10^{-7} M (Wood et al., 1978). A more recent report (McKay & Jameson, 1991) on mMDH using fluorescein-labeled protein and fluorescence-polarization methods indicated that highly active enzyme remained dimeric at 10^{-9} M , and suggested that the disparity in the literature may have been due to the variable specific activities of the different preparations studied. Table 1 summarizes the literature values for the dissociation constant of the mMDH dimer. In view of the discrepancies in the literature, we have re-examined the mMDH system using both steady-state and time-resolved fluorescence methodologies.

Results

The labeling ratio (mol FITC/mol dimeric mMDH) of the mMDH–FITC conjugates varied between 0.1–0.4 for both labeling proto-

Table 1. *Dissociation constants reported for the dimer/monomer equilibrium of mMDH*

Reference	K_D (M)	Technique; conditions
Shore and Chakrabarti (1976)	2×10^{-7}	Fluorescence polarization; 50 mM Tris-acetate, pH 8.0
Bleile et al. (1977)	$>2 \times 10^{-7}$	Gel filtration chromatography and ultracentrifugation; 50 mM sodium phosphate, pH 7.0
Hodges et al. (1977)	pH dependent	Sedimentation velocity ultracentrifugation
Frieden et al. (1978)	$<10^{-9}$	Enzyme kinetics; 50 mM sodium phosphate, pH 7.5
Wood et al. (1978)	pH dependent	Gel filtration chromatography; 50 mM sodium phosphate
Jaenicke et al. (1979)	$<3 \times 10^{-9}$	Gel filtration chromatography
McKay and Jameson (1991)	$<10^{-9}$	Fluorescence polarization; 100 mM phosphate, pH 7.5

cols utilized [the Tris (T) or phosphate (P) protocol: see Materials and methods). The specific activities of these conjugates were essentially the same as that of the unlabeled material, i.e., 1,000–1,200 U/mg, demonstrating that the labeling procedure did not affect the active site of the enzyme. Figure 1 shows the polarization of the mMDH–FITC conjugates (closed circles), prepared using protocol P, in 0.1 M potassium phosphate buffer, pH 8.0, as a function of protein concentration. In this case, the polarization remains near 0.33 over a protein concentration range of 2×10^{-6} to 2×10^{-9} M. The lifetime data for this conjugate (data not shown) fit well to a single Lorentzian distribution with a center value of 4.16 ± 0.05 ns and a width of 0.64 ± 0.17 ns. Dynamic polarization data on this conjugate fit well to two rotational relaxation times, namely, 103 ± 7 ns (ρ_1) and 1.0 ± 0.1 ns (ρ_2), with relative fractional amplitudes of 0.59 and 0.41, respectively, as shown in Table 2. The rotational relaxation times and the fractional amplitudes of each component remain relatively constant over the protein concentration range of 3.8×10^{-6} to 3×10^{-8} M. Sephacryl S-300 chromatography of the mMDH–FITC conjugates at 3.8×10^{-6} M shows a single peak (Fig. 2) at an elution volume of 16.3 mL, which corresponds to a 70 ± 5 kDa molecule (column calibration data not shown).

Figure 1 also shows the polarization, at pH 8.0, as a function of protein concentration, for mMDH–FITC conjugates preparing using

labeling protocol T. Contrary to the previous case, conjugates prepared in this manner displayed concentration dependent variations of the polarization. Each open symbol curve displayed in Figure 1 represents a different mMDH–FITC preparation (T protocol), demonstrating that considerable variation exists between samples. In the Sephacryl S-300 chromatography elution profiles of these conjugates, showed in Figure 2, two peaks can be observed: a peak centered at 16.3 mL, corresponding to a 70 kDa molecule, and a second peak corresponding to high molecular weight aggregations eluting in the dead volume of the column.

The concentration dependence of the polarization of mMDH–FITC conjugates, prepared using protocol P, in 0.1 M potassium phosphate buffer, was observed at pH values of 8.0, 7.0, 6.0, and 5.0. At any given pH value, the polarization was invariant, with concentration down to 1 nm, although the absolute polarization value increased as the pH decreased due to pH-dependent shortening of the lifetime (lifetime data not shown). Specifically, the observed polarizations at pH values 8.0, 7.0, 6.0, and 5.0 were 0.33, 0.35, 0.36, and 0.37; in all cases standard deviations in the polarization values were less than 0.005.

The effect of guanidinium chloride (GuHCl) on the rotational relaxation time (determined using dynamic polarization measurements) of mMDH–FITC, prepared using protocol P, is shown in Figure 3. This plot demonstrates that the average "global" rota-

Table 2. *Data analysis for dynamic polarization measurements of mMDH–fluorescein conjugates at different concentrations*[a]

mMDH–FITC (μM)	ρ_1 (ns)	f_1	ρ_2 (ns)	f_2	χ^2
3.8	98	0.62	1.1	0.38	0.3
1.9	110	0.62	1.1	0.38	0.6
0.95	96	0.61	0.8	0.39	0.9
0.48	97	0.61	1.0	0.39	0.7
0.24	103	0.59	0.8	0.41	0.8
0.14	100	0.59	0.9	0.41	1.6
0.059	107	0.59	1.0	0.41	4.2
0.030	115	0.52	1.2	0.48	2.2
Average	103 ± 7.0	0.59 ± 0.03	1.0 ± 0.1	0.41 ± 0.03	

[a] ρ_1 and ρ_2 correspond to the rotational relaxation times, and f_1 and f_2 are the associated fractional contributions of the rotational relaxation times to the limiting anisotropy $(r_o = 0.37)$.
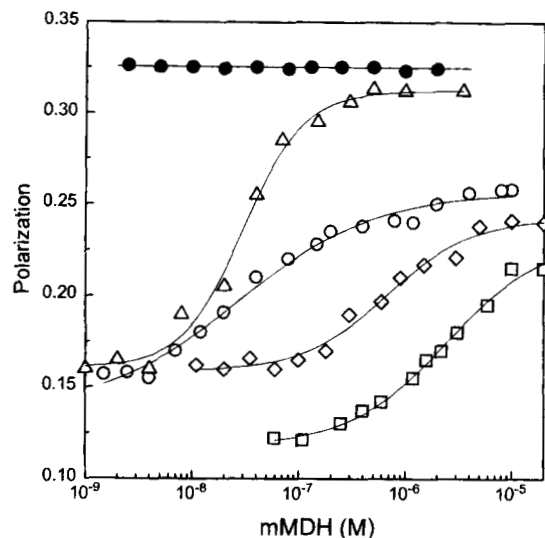
Fig. 1. Polarization of mMDH–FITC conjugates, prepared by both P and T protocols, as a function of protein concentration at pH 8.0. Filled circles correspond to mMDH–FITC prepared using the P protocol. All other symbols correspond to mMDH–FITC prepared using the T protocol.

Fig. 3. Effect of guanidinium hydrochloride on mMDH structure. Change in ellipticity at 222 nm (filled squares) and "global" rotational relaxation time for mMDH-FITC (open circles) at different GuHCl concentrations.

tional relaxation time, obtained from dynamic polarization data, decreases as the GuHCl concentration increases. The same plot also shows the CD signal taken at the same denaturant concentration but using unlabeled protein. A decrease in the CD signal at 222 nm, indicating changes in the secondary structure of mMDH, was observed when the GuHCl concentration exceeded 0.5 M. mMDH–FITC conjugates, prepared using protocol P, were also subjected to elevated hydrostatic pressure. The polarization of these
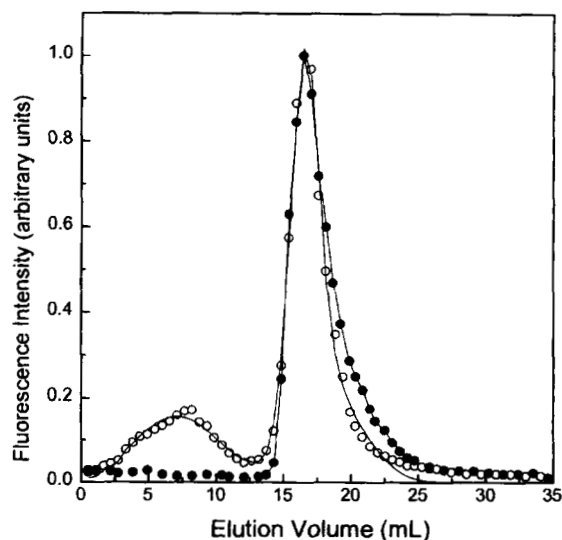
conjugates remained constant at 0.33 from 1 atm to 2,500 bars (data not shown), suggesting that the aggregation state of mMDH–FITC did not change over this pressure range.

Discussion

A starting point for consideration of the lifetime/polarization data on mMDH–FITC systems is the Perrin equation (Weber, 1952):

$$(1/P - \tfrac{1}{3}) = (1/P_o - \tfrac{1}{3})(1 + 3\tau/\rho_h) \qquad (1)$$

where P is the observed steady-state polarization, P_o is the limiting or intrinsic polarization in the absence of polarizing influences such as rotation or energy transfer, τ is the excited state lifetime, and ρ_h the harmonic mean of the Debye rotational relaxation times about the principal axes of rotation. ρ_h is defined as

$$\rho_h^{-1} = (\rho_1^{-1} + \rho_2^{-1} + \rho_3^{-1})/3 \qquad (2)$$

where ρ_1, ρ_2, and ρ_3 are the rotational relaxation times about the principal rotation axes. For a spherical molecule $\rho_1 = \rho_2 = \rho_3 = \rho_0$ and

$$\rho_o = \left(\frac{3\eta V}{RT}\right) = \left(\frac{3\eta M(\bar{v} + h)}{RT}\right) \qquad (3)$$

where η is the medium's viscosity, V the molar volume monitored by the probe, R the gas constant, T the absolute temperature, v the partial specific volume, h the degree of hydration, and M the molecular weight. Using the Perrin equation and the observed polarization of 0.33 (Fig. 1) for mMDH–FITC conjugates prepared using protocol P, a P_o value for fluorescein of 0.47 (Hamman et al., 1996) and the observed lifetime of 4.16 ns, the calculated rotational relaxation time calculated is 25 ns. The Debye rotational

Fig. 2. S-300 elution profiles. Elution profile for mMDH–FITC conjugates prepared using the P (closed circles) and T (open circles) labeling protocols, eluted through a S-300 column with dimensions 25 × 1.2 cm and 0.44 cm/mL flux. The peak centered at 16.3 mL corresponds to a protein of 70 kDa, and the peaks centered between 5 and 8 mL correspond to proteins eluting in the dead volume of the column.
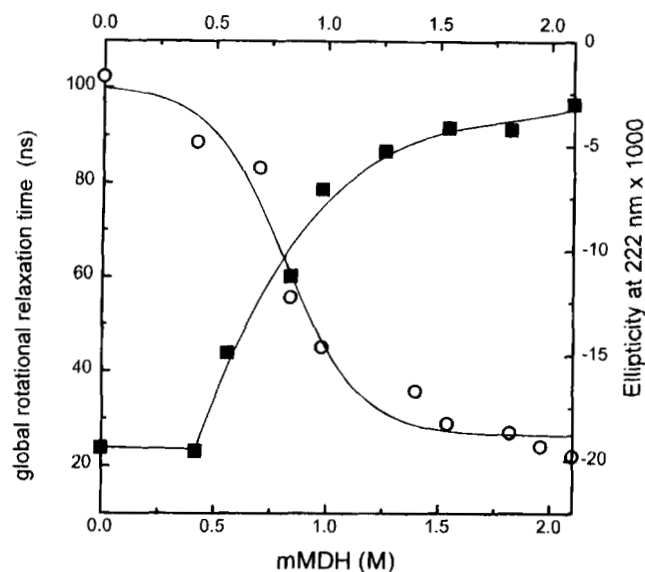
relaxation time expected for a rigid, spherical 68 kDa protein, in aqueous buffer at 20 °C ($\eta = 1.00$ cp), assuming a partial specific volume of 0.75 mL/g [calculated from the amino acid sequence (Birktoft et al., 1982) using the method of Cohn and Edsall (1943)] and a hydration of 0.22 mL/g, is 74 ns. Clearly, the experimentally determined rotational relaxation time is significantly shorter than that expected for a 68 kDa spherical protein, and we may presume that the probe is monitoring more than one rotational motion. The assignment of the terms "global" and "local" motion is somewhat arbitrary. Originally, "local" motion was attributed to mobility of a probe in excess of that expected by the rotation of a rigid body to which it is attached, due to probe motion around its point of attachment to the macromolecule (Wahl & Weber, 1967). Eventually, however, improved methodologies and a better understanding of the dynamic nature of proteins led to the appreciation that "local" motion could include internal or domain motions of the protein as well, such as the segmental flexibility attributed to antibodies (Reidler et al., 1982; Oi et al., 1984; Hamman et al., 1996). Hönes et al. (1986), using NADH as the bound fluorophore, reported a rotational correlation time (ϕ) of 27 ns for mMDH at 25 °C, which corresponds to a Debye rotational relaxation time of 81 ns ($\rho = 3\phi$; Jameson & Sawyer, 1995). They interpreted their results as evidence for a spherical mMDH molecule. Jameson et al. (1989), however, using both steady-state and time-resolved methods on NADH/mMDH adducts, found Debye rotational relaxation times in the range of 106–109 ns, which are more consistent with a nonspherical mMDH molecule. In fact, X-ray crystallographic analysis (Roderick & Banaszak, 1986) has indicated that the mMDH dimer is an ellipsoid with 2:1 axial ratio. These value agree, within experimental error, with the "global" rotational relaxation time (103 ns) determined in this study for the FITC–mMDH adduct prepared using protocol P. Moreover, experimentally determined rotational relaxation times on nonspherical molecules depend upon the orientation of the excitation and emission dipoles of the probes with respect to the principal rotational axes of the macromolecule (Beechem et al., 1986; Brunet et al., 1994) and the differences in rotational rates between probes attached at different sites may reflect these orientational differences.

Table 1 summarizes studies aimed at determination of the dissociation constant for the dimer/monomer equilibrium of mMDH. Our results indicate that it is possible to obtain different K_d values for different protein preparations: 2.2×10^{-6} to 2.4×10^{-8} M (Fig. 1) for mMDH–FITC prepared using protocol T. If the high molecular weigh aggregations contained in this samples (Fig. 2) are removed (protocol P), both the value of steady-state polarization (Fig. 1) and the dynamic polarization results remain constant, clearly demonstrating that the dissociation constant of this enzyme must be tighter than nanomolar. Some of the discrepancy in the literature surrounding the dissociation of mMDH may thus be due to the tendency of the native protein to aggregate under some experimental conditions. Another relevant observation is the report by Place and Benyon (1982) that the elution parameters of mMDH on gel filtration columns depend upon the ionic strength, specifically that at low ionic strengths (<100 mM) the enzyme is retarded by interaction with the gel.

Hydrostatic pressure has been shown to dissociate many oligomeric as a consequence of the lower molar-free volume of the isolated subunits compared to the aggregated proteins (Silva & Weber, 1993). Elevated pressure shifts equilibria, such as dimer–monomer equilibria, by reducing the free energy of subunit association by an amount equal to $P\Delta V$, where P is the pressure and ΔV

is the change in molar-free volume upon dissociation. In the present case, no change in the oligomeric state of mMDH–FITC was observed up to 2.5 kilobars. This result suggests that either the change in molar volume of mMDH, upon dissociation, is very small or that the intrinsic free energy of association is very large (or both).

The pH dependence of the dimer/monomer transition of mMDH has been addressed by Harrison and his collaborators (Bleile et al., 1977; Wood et al., 1978, 1981). Their findings, in part, have motivated other groups to investigate the subunit interface of mMDH using site-directed mutagenesis. Steffan and McAlister-Henn (1991), for example, have studied the pH dependence of the activity of a H46L substitution in mMDH, and demonstrated that this variant has a dramatic shift in the pH profile toward lower pH values, i.e., it has a lower pH optimum for catalysis. They suggest that His-46 may thus be the residue responsible for the pH-dependent dissociation of mMDH. In our view, this conclusion may have to be reconsidered, since we failed to find a pH-dependent dissociation. Ultimately, direct dimer dissociation experiments would have to be carried out on such site-directed mutants to determine the effect of the amino acid substitution on the free energy of subunit association.

Material and methods

Preparation of the mMDH–FITC conjugates

Porcine heart mitochondrial malate dehydrogenase was purchased from Amersham Science Inc. (Cleveland, Ohio). Fluorescein isothiocyanate (FITC) was from Sigma (St. Louis. Missouri), and all other chemicals were analytical grade from Merck (Hawthome, New York). The mMDH was conjugated with the FITC using two different labeling protocols—a phosphate protocol, and a Tris protocol, termed "P" and "T." In the P protocol, lyophilized mMDH powder was dissolved in 0.1 M potassium phosphate buffer, pH 8.0, to a concentration between 3 and 4×10^{-5} M, and desalted, at room temperature, through a NAP-5 column (Sephadex G-25 from Pharmacia Inc., Piscataway, New Jersey) in the same buffer. The eluted sample was incubated with a 20-fold molar excess of FITC at 4 °C and allowed to react for a maximum of 4 h. Free FITC was removed by the NAP-5 column using the same buffer system. In the T protocol, the conjugates were made using 50 mM Tris-acetate buffer at pH 8.0. Lyophilized mMDH was dissolved in buffer to a concentration of 10^{-5} M, incubated with a fivefold molar excess of FITC and allowed to react for 24 h at 4 °C. The free FITC was removed using the NAP-5 column. The fluorescein labeled enzyme was then dialyzed at 4 °C for several hours against 50 mM Tris-acetate buffer, pH 8.0. Protein concentrations were determined using the Bradford method (Bradford, 1976), and labeling ratios of the mMDH–FITC conjugates were calculated using a molar extinction coefficient for fluorescein of $\epsilon_{499} = 70,000$ M^{-1} cm^{-1} (Jablonski et al., 1983).

Enzymatic activity

The standard assay medium used was 100 mM phosphate buffer pH 7.0. The assay was carried out at 25 °C. The substrate concentrations were: NADH 10 mg/mL and 10 mM oxaloacetate. The activity was followed in the direction NADH $\rightarrow$ NAD+ by monitoring the decrease in absorbance at 340 nm using a Genesis 200 Spectrophotometer, using an extinction coefficient for NADH of $\epsilon_{340} = 6,200$ M^{-1} cm^{-1}.

Size-exclusion chromatography

The exclusion column, with dimensions of 25×1.2 cm, was filled with Sephacryl S-300 (Pharmacia Biotech, Piscataway, New Jersey) suspended in 50 mM Tris-acetate buffer pH 8.0. The column was eluted with 50 mM Tris-acetate pH 8.0, at a flow rate of 0.44 mL/min. The molecular weight standards utilized were cytochrome c (12 kDa), carbonic anhydrase (30 kDa), lactate dehydrogenase (150 kDa), and β-amylase (200 kDa). The column void was determined using Dextran Blue.

Circular dichroism experiments

Circular dichroism spectra were taken on a JASCO J-720 spectropolarimeter. Spectra were obtained for mMDH under varying guanidine hydrochloride concentrations at a protein concentration of 2×10^{-6} M in 100 mM phosphate buffer, pH 8. Samples were incubated with denaturant for 1 h before spectra were collected. Each spectrum was obtained at 25 °C in a 0.1-cm optical path cell. Wavelengths were scanned between 250 to 190 nm at 50 nm/min, with a bandpass of 0.5 nm.

Steady-state and time-resolved fluorescence

Steady-state and time-resolved measurements were obtained on a ISS Greg 200 multifrequency phase and modulation spectrofluorimeter (ISS, Inc., Champaign, Illinois) using the 488-nm line of an Argon Ion laser (Technology ILT Model 5490A, Salt Lake City, Utah). Emission at wavelengths greater than 520 nm was viewed through a Schott 083 cuton filter. To eliminate polarization effects, lifetime data were collected under magic angle conditions, in which the excitation beam was polarized normal to the laboratory plane and the emission was viewed through a polarizer set to 55° (Spencer & Weber, 1970). Phase and modulation values for both lifetime and dynamic polarization data were collected in the standard manner (Spencer & Weber, 1969; Jameson et al., 1984; Jameson & Hazlett, 1991). The lifetime data were analyzed either by assuming a sum of discrete exponentials (Jameson et al., 1984) or using continuous distribution models that assumed Lorentzian distributions (Alcala et al., 1987a, 1987b, 1987c) and the goodness of the fit to a particular model was judged by the value of the reduced chi-square (χ^2). A constant, frequency-independent standard deviation of 0.2° for phase and 0.004 for modulation were used in the analysis.

The multifrequency phase and modulation method also permit characterization of the rotational modes of fluorophores using differential polarized phase fluorimetry also known as dynamic polarization (Gratton et al., 1984; Jameson & Hazlett, 1991). In this approach, the excited light, modulated at varying frequencies, is polarized parallel to the laboratory axis, and the phase delay between the perpendicular and parallel components of the emission is determined as well as the ratio of their AC components. The theoretical expressions describing the dynamic polarization data, which is the frequency domain equivalent of anisotropy decay, are complex and described in the literature (Gratton et al., 1984). Here, for clarity, we discuss the data analysis in terms of the time domain. The time-resolved anisotropy of a fluorophor conjugated to a protein contains contributions from the overall rotational diffusion of the protein (global motion) as well as from motions of the fluorophore at its point of (local motion). If the local motion is assumed to be significantly faster than the global motion, the anisotropy decay can be

described by the simple double exponential expression shown in Equation 1 (Gratton et al., 1984; Jameson & Hazlett, 1991), and in the simplest, limiting case, in which $\rho_1 \gg \rho_2$:

$$r(t) = r_0[f_1 \exp(-3\tau/\rho_1) + f_2 \exp(-3\tau/\rho_2)] \qquad (4)$$

where r_o is the time zero anisotropy (the limiting anisotropy; fixed in our analysis to 0.37, which corresponds to a P_o value of 0.47), τ is the fluorescence lifetime, ρ_1 and ρ_2 are the Debye rotational relaxation times associated with the "global" and the "local" rotations, respectively, and f_1 and f_2 are the fractional changes in anisotropy associated with ρ_1 and ρ_2, respectively. This equation was converted to the frequency domain by the method outlined by Weber (1977) and fits were performed using a nonlinear analysis. Frequency independent standard deviations of 0.2° and 0.004 for phase and modulation, respectively, were used.

Acknowledgments

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