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Solvation Dynamics in Protein Environments: Comparison of Fluorescence Upconversion Measurements of Coumarin 153 in Monomeric Hemeproteins with Molecular Dynamics Simulations

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Abstract
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Keywords
Proteins, Emission spectra, Molecular dynamics, Fluorescence, Solvents

Comments
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Solvation dynamics in protein environments: Comparison of fluorescence upconversion measurements of coumarin 153 in monomeric hemeproteins with molecular dynamics simulations

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The complexes of the fluorescence probe coumarin 153 with apomyoglobin and apoleghemoglobin are used as model systems to study solvation dynamics in proteins. Time-resolved Stokes shift experiments are compared with molecular dynamics simulations, and very good agreement is obtained. The solvation of the coumarin probe is very rapid with approximately 60% occurring within 300 fs and is attributed to interactions with water (or possibly to the protein itself). Differences in the solvation relaxation (or correlation) function $C(t)$ for the two proteins are attributed to differences in their hemepockets. © *2007 American Institute of Physics*.
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INTRODUCTION

It has been well established by numerous experimental and theoretical studies that solvation dynamics in polar solvents can be described by the linear response theory.[1–4] In general, the full frequency dependent dielectric function of the polar solvent (and, perhaps, even of ionic solvents[5]) gives a good description of the solvation dynamics from the ultrafast regime to that of diffusive relaxation. Some direct and successful comparisons between theory and experiments have been established.[1,4–6] Such success is achieved largely because the dielectric fluctuations of polar solvents can be described accurately by simple linear response models, such as the dielectric continuum model.[7,8] On the other hand, the structure and function of a protein are determined by a delicate balance of different interactions, mainly of noncovalent nature. Among these, the correct description of electrostatic interactions is critical in the understanding of protein properties. To date, much effort has been put to the investigation of their static role in the structure and function of a protein, and considerable progress has been made with this approach for the analysis of structural stability, molecular recognition and drug design, the efficiency of enzyme catalysis, and other properties.[9] For many elementary processes occurring in a protein its dynamical dielectric response is also important. A prime example is that electron and energy transfers in photosynthesis are modulated by the dielectric medium of a protein complex.[7] Studies of these dynamical responses have been a very active field, both theoretically and experimentally;[10–18] but in spite of considerable efforts towards the understanding of the dielectric relaxation processes in proteins,[2,19] up to now a reliable estimate for the dielectric response function of proteins is still lacking.

For example, a range of experiments has been performed to study dielectric relaxation in proteins, but the results have been very disparate. Early studies suggested that slow relaxation, on the nanosecond time scale, exists in myoglobin,[10] in contrast to polar solvents. This may not be unexpected owing to structural constraints, but the role of a protein's interior motions in its dielectric relaxation is presently unclear from various experimental studies.[11,12,14] Homoelle *et al.* have suggested that the dynamical fluctuations observed in phycobiliproteins involve the interior motions of the protein substantially.[11] Fraga and Loppnow[20] have shown that the resonance Raman spectra are affected by the different residue compositions of the blue copper proteins from different species. On the other hand, experimental and theoretical studies of lysozyme suggest that significant contributions of the observed dynamical fluctuations come from the surrounding water solvent and the water molecules attached on the protein surface.[12] As another example, Zewail and coworkers used tryptophan as a probe to study solvation dynamics in proteins[14–18,21] and have reported slow relaxation from which they inferred the presence of "biological water." Water molecules in the immediate vicinity of a surface believed to have different properties from those of bulk water.[2,22] For example, they report that the dynamics are significantly slower for the surface tryptophan residues in Subtilisin Carlsberg[16] and in monellin[17] than for that of tryptophan in bulk water, and they argue that the slow relaxation arises from the water molecules constrained on the protein surface.[14] The changes in fluorescence emission maxima that

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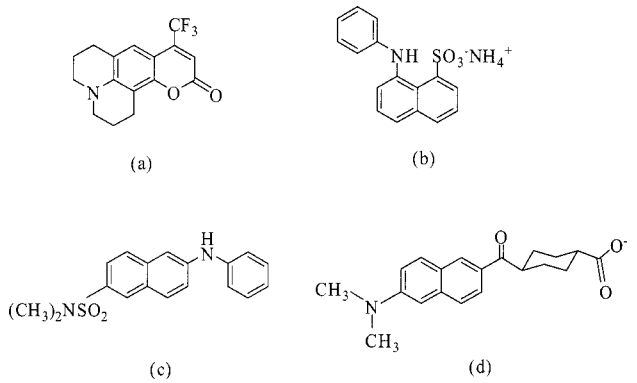

FIG. 1. Structures of the fluorescent probe molecules: (a) coumarin 153 (C153), (b) the ammonium salt of 8-anilino-1-naphthalenesulfonic acid (1,8-ANS), (c) anilino-2-aminonaphthalene-6-dimethylsulfonamide (2,6-ANSDMA), and (d) 2′-(N,N-dimethylamino)-6-naphthoyl-4-*trans*-cyclohexanoic acid (DANCA). The structures for ANSDMA and DANCA were incorrectly transmitted in Ref. 25.

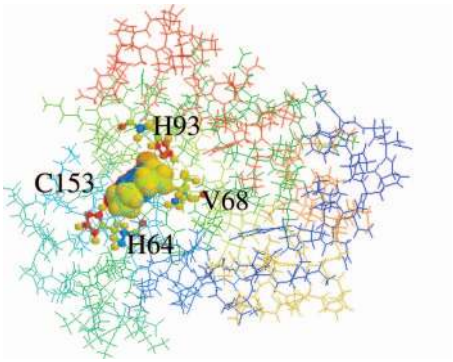

FIG. 2. (Color) A snapshot of equilibrated C153-apomyoglobin in water from 3 ns molecular dynamics simulations using CHARMM22 force field. The C153 is shown in a space-filling model, and two histidine residues in the hemepocket are also shown with stick and ball models. His93 is the proximal histidine belonging to the F helix and is also referred to as HisF8. His64 is the distal histidine, and also referred to as HisE7.

they report for Subtilisin and monellin are, however, 1440 and 960 cm^{-1}, respectively. Given this difference of 480 cm^{-1} for the two surface tryptophans, it would seem that there is also a considerable relaxation arising from the different amino acids neighboring them.

These differences in the interpretations of various experiments are in no small part due to the lack of a reliable dielectric response function for the studied proteins from either experiments or computer simulations. Studies of the solvation dynamics in proteins, nevertheless, offer the best means of investigating the dielectric response. In this work, we discuss the solvation dynamics of the complexes of coumarin 153 (C153) (Fig. 1) with the monomeric hemeproteins, apomyoglobin, and apoleghemoglobin, in water. There are four main considerations for the choice of this system.

First, coumarin 153 (C153) is a well characterized and widely used chromophore for solvation dynamics studies.[23,24]

Second, we have experimentally obtained a binding constant of ~6 μM for coumarin 153 and apomyoglobin and have characterized the complex.[25,26] In fact, one of our motivations for using coumarin to probe the hemepocket was the existence of a NMR structure of the dye ANS, a molecule similar to coumarin (Fig. 1), in the hemepocket of apomyoglobin.[27] Binding studies based upon a Job's plot analysis, circular dichroism, fluorescence depolarization, capillary electrophoresis, and molecular dynamics simulations indicate that coumarin indeed is in the hemepocket (Fig. 2). Furthermore, the coumarin's rotation in the hemepocket is very slow compared to the relaxation time scale of interest (see Fig. 2 of Ref. 25). Finally, for the H64Y/V68F double mutant of myoglobin the reorganization energy increases by 5 cm^{-1} and for the H64W mutant it decreases by 90 cm^{-1}, on the other hand, a surface mutant D112N, has, within experimental error, the same reorganization energy as the wild type. This confirms the presence of coumarin in the hemepocket as opposed to the surface.

Third, while myoglobin and leghemoglobin share a common globin fold, they have differences in their hemepockets,[28] the region to be probed by the coumarin. For example, the F helix is oriented in such a way that in myoglobin HisF8 (His93) eclipses the pyrrole nitrogens of the porphyrin but in leghemoglobin it is staggered with respect to them. In the myoglobin proximal hemepocket, SerF7 facilitates a hydrogen bonding network that drives HisF8 into a conformation that destabilizes ligand affinity. The opposite is true in leghemoglobin, which lacks SerF7 and contains a proximal hemepocket that destabilizes ligand binding. The two proteins exhibit differences on the distal sides of their heme pockets as well. The leghemoglobin distal pocket is larger and more flexible than those of most other hemoglobins and contains a combination of HisE7 (His64) and TyrB10 not found naturally in any other hemoglobin.

Fourth, we can produce a broad range of mutant proteins in which one or several amino acids are strategically replaced, so as to test how specific substitutions can affect solvation dynamics.

MATERIALS AND METHODS

C153 was purchased from Exciton Inc. (Dayton, OH) and used without further purification. Horse heart myoglobin (Mb) was purchased from Sigma. Apoproteins were prepared using a method described elsewhere.[29] C153 has very low solubility in water. A stock solution of C153 was prepared by adding a microliter amount of a concentrated solution of C153 in methanol to water. That is, concentrated C153/MeOH was added to water, keeping the organic content $<0.3\%$ (v/v) in the final solution. To prepare a 5×10^{-5} *M* solution of C153/water, 5 μl of 20×10^{-3} *M* C153/MeOH solution was added to 2 ml of water. The resulting solution was sonicated. For fluorescence upconversion experiments a stock solution of C153/MeOH was added to 1.2 ml of $\sim1.2\times10^{-3}$ *M* apoprotein solution keeping the organic content $<3\%$ (v/v) in the final solution with 1:1 protein to C153 ratio. All samples were equilibrated for about 2 h before taking the steady state and time resolved measurements. For soybean leghemoglobin (Lba), ammonium sulfate was added to 30% saturation and centrifuged at 14 000 rpm for 10 min. The protein in the supernatant was then precipitated by slowly adding ammonium sulfate (to avoid local denatur-

ation) to 90% saturation followed by centrifugation at 14 000 rpm for 10 min. The pellets were resuspended in 20 mM tris buffer, *p*H of 8.0, and then loaded onto a phenyl sepharose (Sigma) column which was preequilibrated with 2 *M* ammonium sulfate in 20 mM tris buffer, *p*H of 8.0. The protein was eluted with 0.4 *M* ammonium sulfate. The eluted protein was dialyzed into 20 mM tris buffer, *p*H of 8.0. The dialyzed protein was loaded onto a DEAE (Phramacia column) and eluted with 75 mM NaCl in 20 mM tris buffer, *p*H of 8.0. The eluted protein was concentrated to ~1 mL and then it was run through a size exclusion column and washed with 10 mM phosphate buffer, *p*H of 7.0.

Steady-state measurements. Steady-state absorbance spectra were obtained on a Hewlett-Packard 8453 UV-visible spectrophotometer with 1 nm resolution. All samples were prepared in 10 mM phosphate buffer solution. The concentrations of apoproteins were determined spectrophotometrically using the extinction coefficient of 15.2 mM^{-1} cm^{-1} at 280 nm.[25] Steady-state fluorescence spectra were obtained on a Spex Fluoromax-2 with a 4 nm bandpass and corrected for lamp spectral intensity and detector response. For both fluorescence and absorption measurements, a 3 mm pathlength quartz cuvette was used. The adequacy of the correction factors and the calibration of our fluorometer were checked against the tabulations of Gardecki and Maroncelli.[30]

Time-resolved measurements. The apparatus for fluorescence upconversion measurements is described elsewhere.[31] The instrument response function had a full width at half maximum of 300 fs. A rotating sample cell was used. To construct the time-resolved spectra from upconversion measurements, a series of decays were collected typically from 480 to 560 nm at 10 nm intervals. Transients were fit to sums of exponentials, and time-dependent spectra were reconstructed from these fits by normalizing to the steady-state spectra,

$$S(\lambda,t) = D(\lambda,t)\frac{S_0(\lambda)}{\int_0^\infty D(\lambda,t)dt}, \tag{1}$$

$D(\lambda,t)$ is the wavelength-resolved fluorescence decay, and $S_0(\lambda)$ is the steady-state emission intensity at a given wavelength. We have employed the traditional approach of fitting the time-resolved spectra to a log-normal function,[23,31] from which we extract the peak frequency $\nu(t)$ as a function of time.

The solvation dynamics were described by the following normalized correlation function:

$$C(t) = \frac{\nu(t) - \nu(\infty)}{\nu(\text{“}t=0\text{”}) - \nu(\infty)}, \tag{2}$$

$\nu(\text{“}t=0\text{”})$ is the frequency at “zero time.”[5,32,33] $\nu(\infty)$ is (usually[34]) the frequency at “infinite time,” the maximum of the steady-state fluorescence spectrum. $\nu(t)$ is determined by taking the maxima from the lognormal fits as the emission maximum. In most of the cases, however, the spectra are broad, so there is some uncertainty in the exact position of the emission maxima. Thus, we have considered the range of the raw data points in the neighborhood of the maximum to estimate an error for the maximum obtained from the lognormal fit. Depending on the width of the spectrum (i.e., zero-time, steady-state, or time-resolved emission spectrum), we have determined the typical uncertainties as follows: zero-time ~ steady-state ($\sim\pm100$ cm^{-1}) < time-resolved emission ($\sim\pm200$ cm^{-1}). We use these uncertainties to compute error bars for the $C(t)$. Finally, in generating the $C(t)$, the first point was obtained from the zero-time spectrum. The second point was taken at the maximum of the instrument response function. The fractional solvation at 300 fs is given by $f(t=300\text{ fs}) = 1 - C(t=300\text{ fs})$.

Molecular dynamics simulations. The starting configurations of horse heart myoglobin and leghemoglobin are from the protein DATA BANK (PDB id 1WLA and 1BIN) with TIP3P water models. To have a reasonable starting point for the C153-protein complex the heme is replaced by C153 and then energy minimization is used to obtain the starting configuration of the C153-protein complex. Standard constant pressure-temperature molecular dynamics was performed using the ORAC package[35] with the Amber force field.[36] In all simulations, short-range nonbonded interactions were calculated up to a 10 Å cutoff, whereas long-range electrostatic interactions were treated by the smooth particle mesh Ewald (SPME) method using a very fine grid, 128 points per axis, with periodic boundary conditions, and an Ewald convergence parameter of 0.43 $Å^{-1}$. Three different Nosé-Hoover thermostats were coupled to the solute, solvent, and total center of mass. An external pressure of 0.1 MPa was applied all along the trajectory. A five time-step rRESPA (Ref. 37) algorithm with times of 0.5–1.0–2.0–4.0–12.0 fs was used with bond constraints on hydrogen covalent bonds handled by a Shake-Rattle-type algorithm. The final system was first equilibrated with velocity rescaling for 60 ps at 50 K and 80 ps at 300 K. Following this initial equilibration, we ran the system for one additional nanosecond at constant temperature ($T=300$ K) and pressure ($P=0.1$ MPa). To achieve full relaxation, the simulation box was entirely flexible for the first 300 ps, whereas for the remainder of the run only isotropic changes of the box were allowed.[35] Finally, the system was simulated for an additional 10 ns. As we have demonstrated in our previous work,[25] an equilibrium configuration for C153 in the hemepocket of the protein can be found and experimental measurements seem to support our interpretation. Using the equilibrated configuration, additional 12 ns trajectories are generated and are used for the calculation of solvation correlations functions.

Using the charges of C153 in the ground and excited states,[4] the solvation correlation function can be obtained within the linear response theory[38] as

$$C(t) = \frac{\langle \delta\Delta E(t)\,\delta\Delta E(0)\rangle}{\langle \delta\Delta E(0)\,\delta\Delta E(0)\rangle}, \tag{3}$$

where $\delta\Delta E(t) = \Delta E(t) - \langle\Delta E(t)\rangle$, and $\Delta E(t)$ is the interaction energy difference between C153 in its excited state and ground state with surrounding protein and water molecules at time t. The symbol $\langle\cdots\rangle$ denotes the ensemble average in the simulation.

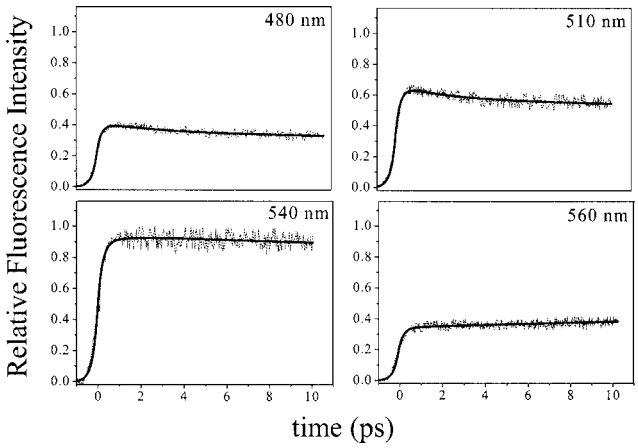

FIG. 3. Fluorescence upconversion traces obtained for C153 in apoMb at the indicated wavelengths. The maximum intensity of the traces are relative to the most intense, i.e., that at 540 nm. The decays used to construct the time-resolved emission spectra were typically collected over a range of wavelengths from 480 to 560 nm at 10 nm intervals; a total of eight or nine decays was used to generate the time-resolved emission spectra, from which the $C(t)$ were calculated.

RESULTS

Representative wavelength resolved traces obtained on an ~10 ps time scale by means of fluorescence upconversion are shown in Fig. 3. Figure 4 provides the time-resolved emission spectra at 300 fs and 10.3 ps along with the steady-state and zero-time spectra. Figure 5 presents the solvation correlation functions $C(t)$. The $C(t)$ obtained from molecular dynamics simulations are also compared with the experimental data in Fig. 5. Relevant fitting parameters are summarized in Table I. The salient results are

(1) Almost 60% of the solvation is complete in both apoMb and apoLba within the time resolution of our instrument (300 fs).

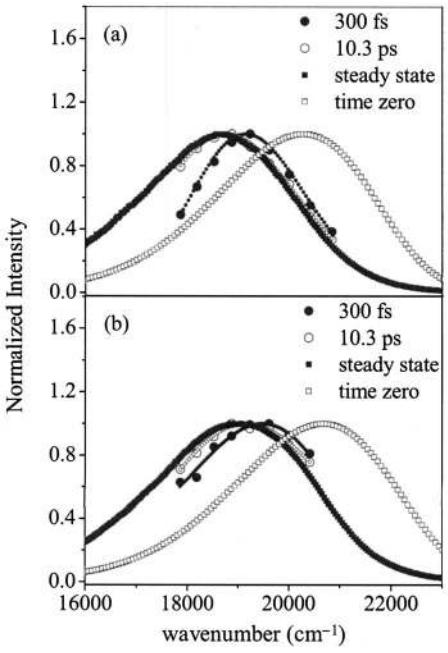

FIG. 4. Normalized time resolved emission spectra for C153 in (a) apoMb and (b) apoLba at 300 fs and 10.3 ps. Corresponding steady-state and "zero-time" spectra are included. Almost 60% of the solvation is complete in both systems within the time resolution of our instrument (300 fs).

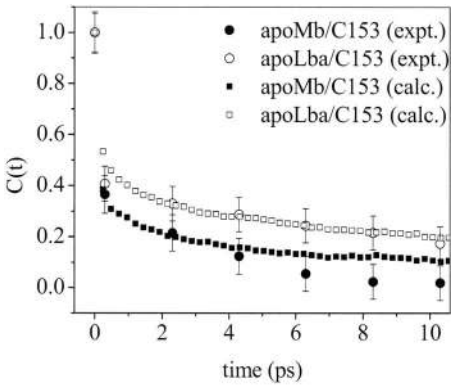

FIG. 5. Comparison of $C(t)$ for C153 in apoMb and apoLba obtained from fluorescence upconversion experiments with those obtained from molecular dynamics simulations. In both proteins, the initial fast component occurs within the time resolution of our instrument.

(2) The initial faster solvation is followed by a slower response, which is slower in apoLba than in apoMb by about a factor of 4 (Table I).

(3) There is excellent agreement between the $C(t)$ from fluorescence upconversion experiments and those obtained from molecular dynamics simulations.

DISCUSSION

The rapidity of the solvation in both the proteins studied here suggests that water is playing a dominant role, which is consistent with the report by Jordanides *et al.*[12] that solvation in the lysozyme/eosin system is dominated by water. (Solvation in bulk water is characterized largely by an ~30 fs component and is complete in ~15 ps.[6,39]) The remainder of the solvation can be attributed to motions of the protein matrix or coupled protein-water[40] motions. The protein's contribution to solvation should not be neglected. For example, Nilsson and Halle have simulated the Stokes shift in the protein monellin[41] and have discussed how to separate the relative contributions of protein and water. They found a significant protein component, at least 25%. Li *et al.*[40] found that the relative protein and water contributions can vary substantially with the conformational substate of myoglobin: Sometimes the protein contribution can even be larger than water. Both Nilsson and Halle[41] and Li *et al.*[40] found that the protein contribution also has an ultrafast component. Li *et al.* also found that, in disagreement with the "biological water" picture, protein motion (or protein-water motion) was essential for the slow (~50–100 ps) time-scale Stokes shifts. This feature was independent of the dynamics apparent from the protein and water Stokes shift contributions.

Our results are, however, at odds with those of previous attempts to exploit the myoglobin system to study the solvation response of proteins. These studies[10] used the fluorescent probes, 2,6-ANSDMA, and DANCA (Fig. 1). The former probe molecule afforded a single exponential response of 9.1 ns; the latter is a more complicated response with both shorter and longer response times. The discrepancy

TABLE I. Solvation of C153 in two hemeproteins (20 °C). Unless otherwise indicated, the parameter refers to that experimentally obtained. The time constants are in picoseconds and the frequencies and reorganization energies are given in wave numbers.

System	$f_{300\ \mathrm{fs}}$	a_1[a]	τ_1	τ_2	a_1^{calc}	τ_1^{calc}	τ_2^{calc}	$\langle\tau\rangle$	$\langle\tau\rangle^{\mathrm{calc}}$	$\nu(\text{“0”})$[b]	$\lambda(\text{“0”})$[c]	$\lambda(\infty)$[c]
apoMb	0.64	0.59	0.02	3.4	0.73	0.14	9.3	1.4	2.6	20 260	1850	2450
apoLba	0.59	0.60	0.09	13	0.60	0.19	13	5.3	5.3	20 660	1840	2590

[a]The solvation relaxation function $C(t)$ are in both cases fit to a sum of two decaying exponentials $C(t)=a_1\exp(-t/\tau_1)+a_2\exp(-t/\tau_2)$, where $a_1+a_2=1$. $C(t)$ is fit from its value at unity, i.e., starting at "$t=0$"; consequently the early part of its decay is determined by only two points. The τ_1 we report are thus upper limits for the early portion of the relaxation. The average solvation time was calculated according to equation: $\langle\tau\rangle=\Sigma_i a_i\tau_i$.
[b]For apoMb/C153, $\nu(\text{“0”})-\nu(\infty)=1530\ \mathrm{cm}^{-1}$; for apoLba/C153, $\nu(\text{“0”})-\nu(\infty)=1600\ \mathrm{cm}^{-1}$. The "zero-time" spectra were calculated according to the method described elsewhere (Refs. 5, 32, and 33). We use the zero-time *spectrum* in our analysis (Fig. 4) and not any approximation for obtaining its maximum. We use hexane as the nonpolar solvent for the zero-time calculation.
[c]The reorganization energy, discussed elsewhere, (Refs. 12, 31, and 33) at "$t=0$" and at steady state.

between the results for these two probe molecules as well as the predominance of the long-lived response time caused us to search for other probes. We consequently opted for coumarin 153, which not only has been studied in a very wide range of solvents and in the gas phase, but whose excited-state solvation has been demonstrated not to involve any contributions other than those from S_1.[23,24]

Our results are also at odds with those of other studies,[16,18] from which it is suggested that aqueous solvation in proteins is much slower than that in bulk water. This slow solvation is attributed to biological water:[2,22] In restricted environments, water is proposed to solvate on a much slower time scale, tens to hundreds of picoseconds, as opposed to ~1 ps.[2] We note, however, that an accurate determination of $C(t)$ depends upon appropriate values for $\nu(0)$ and $\nu(\infty)$. The latter is usually given by the equilibrium spectrum. This is not, however, true in the case of very slowly relaxing solvents, as have been demonstrated in the case of certain ionic liquids:[34] For example, here the emission spectrum at ~ three times the fluorescence lifetime of the probe is *red-shifted* to that of the equilibrium spectrum. The appropriate value for $\nu(0)$ is not obtained from the emission spectrum obtained immediately upon optical excitation with infinite time resolution, even if such an experiment were possible, but that arising from the spectrum of a vibrationally relaxed excited state that has been fully solvated by its internal motions but that has not yet responded to the surrounding solvent. Fee and Maroncelli[32] have described a robust, model independent and simple procedure for generating this zero-time *spectrum* [$\nu(\text{“0”})$]; and we have checked its validity using a different method for estimating the zero-time reorganization energy.[33] Finally, Li *et al.*[40] have compared experiments and simulations for protein solvation and noted a significant discrepancy between theory and experiment: Namely, a very rapid early relaxation is obtained in the simulations but is absent in the experiments. We suggest, based on our results and others to which we refer, that these authors are, in fact, simulating the solvation appropriately and are, rather, missing the rapid dynamics in their experiment and its analysis.

CONCLUSIONS

The results presented here attest to the utility of coumarin 153 as a probe of protein dynamics, as we have suggested in earlier work.[25,26] Since the late 1980s, coumarin 153 has proved to be the most useful and reliable probe of solvation dynamics, has been exhaustively studied, and has successfully withstood numerous challenges to this title.[23,24] Its priority in this arena can be attributed to its large Stokes shift [crucial for acquiring an accurate estimate of $C(t)$], relative rigidity, nonreactivity in the excited state, and that its spectral features arise from only one electronic state. The solvation relaxation functions $C(t)$ obtained from complexes of coumarin 153 with apomyoglobin and apoleghemoglobin by means of fluorescence upconversion experiments and molecular dynamics simulations are in excellent agreement. The solvation of the coumarin probe is very rapid with approximately 60% occurring within 300 fs and is attributed to interactions with water and possibly the protein. The hemepockets of myoglobin and leghemoglobin differ considerably[28] as we note in the Introduction, and this manifests itself in both the experimental results and the molecular dynamics simulations.

The literature concerning protein dielectric relaxation contains conflicting reports and conclusions. Our results are in good agreement with those obtained by Jordanides *et al.*,[12] who found that the initial solvation dynamics of the lysozyme/eosin complex are identical to those of eosin in bulk water. Our results are, however, rather different from those obtained in other studies. Notably, the dynamics we observe are much more rapid than those reported in other works involving monomeric hemeproteins.[10] We suggest that the probes used, ANSDMA and DANCA (Fig. 2), are not ideal probes of solvation. They are much more flexible than coumarin, and they are likely to undergo excited-state charge transfer reactions, which could seriously complicate the interpretation of solvation dynamics. This class of chromospheres is notable for its dual emission from locally excited and charge-transfer states.[42]

We suggest that owing to their methods of analyzing the Stokes shift data other workers have exaggerated the amount of the slowly relaxing component of solvation that they attribute to "biological water."[2,16,18,22] While it is possible that water molecules may be tightly bound to the protein surface and in this way contribute to slower solvation events, we propose that there is no cogent evidence for excluding the ultrafast solvation from bulk water.

Finally, we stress the importance of accurately obtaining the "zero-time" spectrum. Its knowledge is fundamental to an accurate construction of the solvation relaxation function.

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