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Reverse micelles as a tool for probing solvent modulation of protein dynamics: Reverse micelle encapsulated hemoglobin

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Abstract

Hydration waters impact protein dynamics. Dissecting the interplay between hydration waters and dynamics requires a protein that manifests a broad range of dynamics. Proteins in reverse micelles (RMs) have promise as tools to achieve this objective because the water content can be manipulated. Hemoglobin is an appropriate tool with which to probe hydration effects. We describe both a protocol for hemoglobin encapsulation in reverse micelles and a facile method using PEG and cosolvents to manipulate water content. Hydration properties are probed using the water-sensitive fluorescence from Hb bound pyranine and covalently attached Badan. Protein dynamics are probed through ligand recombination traces derived from photodissociated carbonmonooxy hemoglobin on a log scale that exposes the potential role of both and solvent fluctuations in modulating protein dynamics. The results open the possibility of probing hydration level phenomena in this system using a combination of NMR and optical probes.

Keywords

Reverse micelles; Hemoglobin; Protein dynamics; Solvent slaving; Hydration; Geminate recombination

1. Introduction

Understanding how proteins function on a detailed molecular level is a major objective in biophysics. Characterizing equilibrium structures of proteins does not always provide an adequate molecular level explanation for the observed reactivity even for many of the most extensively studied systems. The influence of protein dynamics is often the key missing element in developing a comprehensive explanation of protein function. Protein function is intimately associated with its dynamical properties. Functionally important protein dynamics appear to be coupled and possibly slaved to the dynamics of the surrounding solvent [1–7] although questions remain as to the validity of the model [8,9], the generality of the model [10] and its applicability when systems are probed on a micro-site specific level using techniques such as NMR [11,12]. Both protein dynamics and solvent dynamics seemingly possess a hierarchical make-up with respect to amplitudes, time scales and temperature

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This paper is dedicated to the memory of Robin Hochstrasser, an inspiring and brilliant mentor who never wavered in his commitment to discovery and his passion for excellence in all endeavors. He will be sorely missed. It is hard to imagine a world without his imposing and dignified presence.

dependences when probed in hemeproteins such as myoglobin and hemoglobin [1,2,8,9,13–16]. The challenging biophysical study of the complex interplay between the different categories of protein dynamics and the various types of solvent motions is an essential aspect in exposing how proteins function. To this end, systematically tuning the hydration properties of proteins becomes an important tool in dissecting out how both bulk water and hydration shell waters impact categories of protein dynamics. The present study builds on our earlier work in which sol–gel and glassy matrices are used to systematically tune both protein and solvent dynamics [13,15–22]. Whereas those approaches have the added benefit of allowing for a wide range of temperatures, spanning cryogenic to ambient or even higher, they are limited with respect to being able to fully characterize the nature of the waters surrounding the protein, especially with respect to dimensionality. Proteins encapsulated in reverse micelles [23–26], the focus of the present study, are much more amenable to this kind of quantitative analysis of the behavior of hydration waters [11,12,25,27–31].

Reverse micelles are unique because they represent nano-size pockets of encapsulated water separated from an organic medium by a surfactant [27,32,33]. The size of the micelles can be tuned by varying the ratio of water to the concentration of the surfactant in the organic solvent [34]. Adding water increases the size of the micelle, permitting an examination of the effect of a systematic enhancement of the number of hydration waters surrounding a confined protein [32,35]. Additionally, biologically relevant osmolytes can also be added to the confined water within the reverse micelles. Thus RM's present a potential tool to evaluate not only the hydration sphere, but also the interplay of confinement and osmolytes on the encapsulated protein, including dynamics and conformation [36].

The present study represents our progress in developing RM's as a tool with which to systematically tune hydration properties as a function of confinement conditions and added osmolytes [19,20]. The general approach is to determine how the hydration shell waters and the functional properties of a given protein respond to systematic changes in confinement/co-solvent conditions. To achieve this objective, suitable probes of both hydration and protein properties are needed. Additionally it is essential to establish that the RM encapsulated proteins retain a functional native conformation. Retention of functionally active conformations for hemoglobin in reverse micelles was a concern given the existing literature indicating the loss of heme from RM encapsulated Hb [37,38].

In the present study, properties of the bulk water phase and the hydration shell waters within the micelle are probed using free and protein associated fluorophores. These probes do not have the resolution and informational content of techniques such as NMR but can be used to monitor the systematic change in certain parameters that permit an assessment of the how the hydration waters are responding to specific perturbations. These probes also allow for comparisons of hydration effects in a wide variety of confinement matrices (e.g. glasses, sol–gels, polyols at cryogenic temperatures) that are not accessible to NMR probing. Thus the RM is system that provides a meeting ground for optical and NMR probing of hydration. The fluorophores chosen have emission spectra highly sensitive to water-dependent changes in the local environment of the probe whether the probe is free or bound to protein. Pyranine, or HPT(AKA HPTS) has two major fluorescence emission bands in the visible [39]. Under appropriate conditions, the ratio of the amplitude of these two peaks can be used as a measure of the relative populations of mobile vs. immobile waters in the solvation sphere of the probe [20,39]. This sensitivity arises from the dissociation/recombination behavior of the single ionizable proton on the hydroxyl group of HPT [40]. The pKa of this hydroxyl in the ground state is ~7 but shifts to 0.1 in the excited state [41,42]. The fluorescence spectrum is dependent upon whether the dissociated proton-ionized probe remains stabilized for the duration of the fluorescence lifetime [41]. The mobility of the surrounding water appears to be a major factor in stabilizing the ionized pair. Decreasing the

population of mobile waters is associated with enhancing the recombination times of the ionized pair and thus favors emission from the non-ionized form of the excited singlet state giving rise to the blue shifted fluorescence peak. Thus the progressive change in the ratio of the amplitude of the red versus blue fluorescence peaks can be used to monitor the progressive change in the mobile versus the less mobile water populations as a function of confinement, added osmolytes, temperature and other physico-chemical parameters [20]. There is an added benefit in that HPT is an effective analog of organic phosphates such as DPG (diphosphoglycerate) that bind to the so-called DPG binding site at one end of the water filled central cavity of hemoglobin. As such it can be used to directly probe the status of the hydration water mobility at that site as a function of varied solvent conditions. The difference in the polarity between Hb-coordinated and free HPT results in differences in the blue shifted emission band (~435 nm versus ~454 nm for free HPT versus Hb-complexed HPT) that allow for the unambiguous determination of the extent of binding under a given set of solution conditions.

Badan (6-bromoacetyl-2-dimethylamino-naphthalene) was chosen as the second fluorescence-based probe of hydration waters. Badan can be covalently bound to a thiol group in peptides or proteins through a reaction with the bromoacetyl moiety of the Badan molecule [43]. The emission maximum is strongly correlated to the polarity and the dielectric relaxation properties of the surrounding environment of the probe [44–46]. In the present study Badan is covalently attached to the thiol on glutathione (GSH), the reactive Cys beta 93 sulfhydryls of HbA and the reactive thiol mutagenically introduced into myoglobin (S3C).

The dynamical properties of hemoglobin and myoglobin have been extensively probed to a much greater degree than almost any other protein system, which continues to make these proteins highly appropriate models to explore the consequences of confinement and other solvent parameters. The recombination traces of nanosecond or faster photodissociated CO from HbA and myoglobin have multiple features that reflect the impact of specific categories of dynamics, and as such can be used as an effective probe of dynamics as a function of solvent conditions [7,18,47,48]. In the present study the effect on the CO recombination trace for an RM encapsulated COHb is probed as a function of enhanced immobilization of the hydration shell waters through progressive elimination of mobile hydration shell waters and the introduction of glycerol. The results are contrasted with those from sol-gel encapsulated COHbA under conditions of added glycerol [13]. The effects of changing both the size of the resulting micelles and the solution components of the encapsulated proteins and peptides, are discussed and extrapolated to *in vivo* confinement issues.

The present study also introduces a new method to systematically fine tune the water content of a fully prepared RM suspension. Large molecular weight PEG (polyethylene glycol) when added to the suspension acts as a potent source of osmotic stress upon the RM's and is shown to progressively pull water from the RM over a sufficiently slow time scale to allow continuous probing of protein and hydration properties during the progressive loss of water.

2. Experimental section

2.1. Materials

Buffer salts, AOT, isooctane, glycerol, tetramethyl orthosilicate (TMOS) and polyethylene glycol were purchased from Sigma (St. Louis, MO) in the purest form available; 3,3,3-trifluoropropyl trimethoxy silane was purchased from Gelest (Morrisville, PA). HPT, Aedans and Badan (BD) were purchased from Molecular Probes (Invitrogen, Grand Island,

NY). Hemoglobin (HbA) was prepared according to previously published procedures (a gift from S. Acharya) [49,50]. Samples were prepared in 0.05 M Bis-Tris, pH 6.5 or 7.0.

2.2. Preparation of fluorescent probe-protein complexes

HbO₂ was solubilized in 0.05 M Bis-Tris, pH 6.5 at a concentration of 2–3 mM. An aliquot of this solution was used to prepare a complex with HPT by adding slightly less than a 1:1 aliquot of HPT to the protein in order to minimize free probe.

In the present study Badan is covalently attached to the reactive Cys beta 93 sulfhydryls of HbA, to glutathione (GSH) and to a reactive thiol mutagenically introduced into myoglobin (Mb) (S3C a gift from Professor R. Callender). Briefly, a concentrated solution of Badan was prepared in DMSO. To a solution of HbO₂, MbO₂ or GSH in 0.05 M Bis-Tris, pH 6.5, an aliquot of the Badan in a 2.5:1 ratio (Badan: Cys) was added with stirring in the cold and in the dark. The reaction was allowed to continue for at least 2 h, at which point the excess Badan was reacted with dithionite, and removed by spin column. Aedans was used to modify hemoglobin in the same manner as Badan.

The structural integrity of encapsulated HbA, Mb, and GSH were assessed using spectroscopic techniques. UV/vis absorption and near and far UV CD were used to establish the status of HbA in the RM; whereas, fluorescence from the Badan was used to evaluate conformational properties of GSH.

2.3. Preparation of sol–gels

TMOS, or TMOS doped with 3,3,3 trifluoropropyl trimethoxy silane was hydrolyzed using 2 mM HCl, and then mixed in a 1:1 ratio with HPT or GSH-BD (modified with Badan) that was solubilized in 0.05 M Bis-Tris OAc buffer, pH 7.0. The mixture was then placed in a cuvette, forming a block [48]. After gelation, the block was bathed with a buffer of choice. Glycerol (100%) replaced the buffer in the gel block. Polyethylene glycol was added to the top of the sol–gel, and replaced periodically.

Hemoglobin bound to HPT or to Badan and the myoglobin mutant modified with Badan were encapsulated in a thin film of silane derived sol–gels prepared as described above. The resulting solution was spun in a 1 cm wide NMR-tube until gelation occurs creating a thin film of an Hb-containing gel on the bottom fourth of the tube. A final concentration of 0.45 mM in heme for the encapsulated Hb or Mb is required to produce a high optical quality thin film.

2.4. Preparation of glasses

A solution was prepared of trehalose or sucrose 0.1 M in water, to which was added an aliquot of HPT from a concentrated stock prepared in 0.05 M Bis-Tris, pH 6.5 [51]. The final concentration of HPT in the sugar solution was 5–10 μ M. This mixture was spread on a glass plate and dried under vacuum for 1–3 days. Cations, at the appropriate concentration, were added to the HPT solution prior to forming the glass. After drying, the glass was heated at 55 °C for 1.5 h.

GSH complexed to Badan was prepared in 0.05 M Bis-Tris pH 7.0 and prepared in glasses in the same way using trehalose or sucrose. The GSH-BD glasses were dried and heated to 40 °C for 1 h.

2.5. Preparation of reverse micelles

Micelles were prepared by weighing an appropriate amount of AOT (0.05–0.1 M) and dissolving it into a premeasured volume of isooctane. The mix was stirred until the solution

was clear. Probe solutions of free HPT (10 μ M) were prepared in buffer (0.05 M Bis-Tris, pH 6.5) and were added to the isooctane/AOT solution to form the micelles and to achieve the desired water:AOT ratio ϕ . (This ratio is the molar ratio of water to surfactant.) In all cases the final solutions were clear. The same volume of liquid was added to the surfactant in each case and included the cosolvents.

For the protein reverse micelles, a concentrated solution of protein (2–3 mM) was solubilized in 0.05 M Bis-Tris, pH 6.5 or 7.0, and added to the AOT/isooctane solution to form the micelle in the appropriate water: AOT ratio. For the solution samples with Badan, the protein was modified with the fluorophore prior to making the micelle. This was also true of protein samples with HPT. Samples with glycerol were prepared with glycerol prior to adding to the AOT/isooctane solution.

2.6. Techniques and methods

Fluorescence Spectra (QuantaMaster Model QM-4/2000SE enhanced performance scanning spectrofluorometer, Photon Technology International, Lawrenceville, NJ) were generated using an excitation wavelength of 355 nm, for HPT and 385 nm for Badan, 1-mm slit width for excitation and emission slits, a 1-cm cell, and an integration time of 0.25 or .5 s at ambient temperature. For HPT, changes in the water mobility were monitored and reported as the ratio of mobile waters to the total population of mobile and immobile waters in the reverse micelle. The values presented compare those of the free and protein-bound probe in solution to those in the micelle using the same solvent conditions. For Badan the main emission peaks were reported.

2.6.1. UV/Vis absorption—Spectra were used to evaluate all samples prepared, as well as fluorophores used in this study. Samples were monitored on a Lambda 2 (Perkin Elmer, Norwalk, CT).

2.6.2. CD measurements—CD spectra (190–480 nm) were recorded with a J-815 spectropolarimeter (Jasco Inc., Easton, MD) at room temperature. COHbA, pH 7.0 Bis-Tris/50% glycerol (volume), was added to the surfactant/organic solution to form the reverse micelle suspension. Samples were measured in a 0.1 cm path length cuvette. Multiple scans were averaged, and baselines were subtracted.

2.6.3. Resonance Raman—Resonance Raman measurements were acquired with the system described previously [52]. Briefly, the 413.1 nm output from a Kr ion laser (Spectra Physics, Mountain View, CA) was focused to a ~ 30 μ m spot on a quartz cuvette rotating at ~ 1000 rpm. The scattered light was collected at a right angle to the incident beam and focused on a 100 μ m entrance slit of a 1.25 m Spex spectrometer equipped with a 1200 grooves/mm grating (Horiba Jobin Yvon, Edison, NJ), where it was dispersed and detected by a liquid nitrogen-cooled charge-coupled device (CCD) detector (Princeton Instruments, Trenton, NJ). The incident laser light was removed by a holographic notch filter (Kaiser, Ann Arbor, MI). The Raman shifts were calibrated by using indene. Cosmic ray artifacts were removed using the *Winspec* software from Roper Scientific (Princeton, NJ). The laser power was 3 mW at the sample for all measurements.

2.6.4. CO recombination—CO recombination following ns photodissociation of COHbA in the reverse micelle was used to evaluate the impact of confinement on functionally important conformational dynamics. COHbA prepared from a stock solution (2.5 mM heme) diluted with glycerol (25% and 50% by volume) was then added to the surfactant/organic mixture comprised of 0.1 M AOT/isooctane to form the reverse micelles. The COHbA samples were photolyzed and probed, generating the full time course from several

nanoseconds out to 1.0 min of the CO recombination trace subsequent to photodissociation using a nanosecond pulse at 532 nm to photodissociate the CO and a weak CW blue output at 441.6 nm from a HeCd laser to monitor the recombination process as described in detail in earlier publications [48,53]. The kinetic traces were compared to previous traces generated from well characterized samples in similar buffer solutions.

3. Results

The results are organized to first present the behavior of the two fluorescent probes of hydration waters, HPT and Badan, in the reverse micelles as a function of added osmolytes and water content. The reverse micelle results are compared with and evaluated within the context of results obtained from the same probes in other matrices exposed to similar perturbations of the water environment surrounding the probe. Whereas in this initial phase of the study, HPT is used alone as an uncomplexed free probe, the corresponding non-protein coordinated Badan results employ this probe covalently bound to the thiol of glutathione, a cysteine containing tripeptide.

3.1. Free-HPT in the reverse micelle

Fig. 1A shows the fluorescence spectrum of HPT in reverse micelles where the water content is progressively diluted through the addition of glycerol. With increasing glycerol content, the fluorescence spectrum evolves from a spectrum characteristic of HPT in aqueous buffer with just the single ~510 nm peak to a spectrum dominated by a blue shifted peak at ~437 nm. Similar changes are observed for sol-gel encapsulated HPT samples as the bathing buffer is progressively doped with higher concentrations of glycerol. [20] Fig. 1B shows how the addition of solid PEG 3 K to the suspension of RM's in isooctane induces a change similar to that seen with the addition of glycerol. Shaking the sample with the added PEG enhances the effect. A similar change is seen for sol-gel samples over a period of many days when an excess of PEG 3 K is placed on the top of an HPT containing sol-gel monolith that has had all of the visible buffer removed. As the spectrum changes, the PEG layer is observed to become progressively wetter consistent with the PEG exerting an osmotic stress on the monolith block which still contains residual water localized in the pores of the sol-gel. In contrast, when comparable amounts of PEG are added to an aqueous solution of HPT, there is no measurable change in the spectrum of the HPT (single band at 510 nm). These changes are also seen for the reverse micelle as the water content is fractionally reduced as part of the preparative protocol (not shown).

In Fig. 1C how, in a glassy thin film derived from trehalose, the addition of cations with a varying capacity for sequestering the limited number of remaining mobile waters, has a large impact on the HPT fluorescence spectrum. As the charge density of the cation increases, the relative fraction of immobile waters decreases, as was observed in solution phase studies in which much higher concentrations of the salts were added [17]. Thus despite the roughly same concentration of water within the glass, the HPT spectrum can reflect differences in the ability of the water populations to stabilize the charge separated excited state singlet of HPT as was also concluded from picosecond lifetime measurements of HPT in reverse micelles [54]. Fig. 1C is presented in part to show the consequence of extreme water immobilization as reflected in the fluorescence spectrum of HPT. As discussed in Section 1, the 510 nm and the 428–450 nm peaks are attributed to emission from the excited state singlet of HPT with the low pKa proton off and on the molecule, respectively, which in the case of aqueous solvents, has been associated with populations of mobile and immobile waters, respectively [20].

3.2. Badan labeled glutathione (GSH-BD) in reverse micelles

Fig. 2 shows the behavior of the fluorescence from Badan coordinated to GSH (GSH-BD) as a function of solution conditions. In Fig. 2A, there is shown a progressive blue shift of the emission peak both as a function of added osmolytes and the combination of osmolytes. The blue shift due to glycerol and trehalose when present separately, appears additive when both are combined. The spectra presented are normalized with respect to intensity. The actual emission intensity increases dramatically as the peak undergoes shifting to the blue [55]. Fig. 2B shows how, in the relatively rigid environment of glasses, derived from either trehalose or sucrose, the emission peak is substantially more blue shifted compared to the solution results shown in Fig. 2A.

The behavior of the Badan fluorescence for GSH-BD in reverse micelles as a function of water content and added osmolytes is shown in Figs. 3A and B, respectively. It can be seen that both the reduction of water content in the preparative phase (3A) and the addition of osmolytes induces blue shifts in the peak position that are much greater than those seen in solution using the same osmolyte additions. The blue shifting in both RM cases is close to that seen for the glass embedded HPT (2B). This observation indicates that the RM enhances the viscogenic properties of the added osmolytes and that the enhanced osmolyte effect is mimicked by merely lowering the water content of the RM. The progressive blue shifting, albeit not to the same extreme as seen in Fig. 3, is also seen for emission from GSH-BD encapsulated in a TMOS derived sol-gel monolith covered with an excess of PEG 3 K (545 nm over a four day period). A similar sol-gel derived from TMOS doped with 3,3,3-trifluoropropyl trimethoxy silane, which imparts a greater degree of hydrophobicity to the gel interior, shows a similar PEG induced effect; however, the starting peak position is already blue shifted relative to the TMOS sol-gel (538 versus 545 nm) as is the final peak position after the four day treatment with PEG (528 versus 532 nm).

3.3. Reverse micelle incorporated hemoglobin-spectra and conformation

The protocol employed to incorporate hemoglobin into the RM yields suspensions that are clear without any evidence of aggregate formation. Similarly clear suspensions are obtained for myoglobin. Fig. 4 shows the absorption spectrum of the carbonmonoxy derivative of Hb (COHbA) incorporated into reverse micelles. In this case the hemoglobin is a chemically modified HbA with a fluorescent dye (Aedans) covalently attached to the thiols on Cys 93. Similar spectra are obtained for the unmodified derivative of HbA. Similar quality absorption spectra are obtained from COMb samples. For both Hb and Mb the spectra from the RM suspensions are essentially the same as those generated from solution. A potential concern is that the RM destabilizes the globin and allows escape of the heme and as a result it is the CO coordinated free heme moiety that is contributing to the absorption spectrum. This possibility was shown to be incorrect based on our generating the CO derivative of free hemin in isooctane and observing that the absorption spectrum of this population is easily distinguished from that of either COHbA or COMb based on the substantially blue shifted Soret band seen for the free hemin sample relative to the protein samples. In contrast, the absorption spectra of both of these proteins in the RM suspension are very similar to that of corresponding samples in aqueous buffer with no indication of peaks corresponding to protein-free heme or changes in the heme structure. Loss of the heme invariably occurs when the native structure of Hb is perturbed. The absence of any detectable free heme is consistent with the CD results showing no detectable alteration in secondary, tertiary or quaternary structure for RM encapsulated Hb or in the position of the heme.

Fig. 5 shows the CD spectrum of an RM incorporated HbA sample. Both the UV and visible portions of the CD spectrum are essentially identical to that of the corresponding solution phase sample [56,57]. There is no evidence [58–63] indicative of either partial unfolding or

change in the helical content of the protein or global tertiary/quaternary conformation. The resonance Raman spectrum, in contrast to the other optical spectroscopic probe techniques, does show a change that reflects a conformational change although it is likely to be highly local. The Sôret band enhanced resonance Raman spectrum from COHbA includes the Raman band assigned to the Fe-CO stretching mode. The frequency of the band is correlated with the conformational status of the distal heme pocket [64,65]. In particular, the conformation sensitive interaction of the imidazole side chain of the distal histidine greatly impacts the frequency [66]. In an aqueous solution of COHbA at pH 6.5 with glycerol added at a 12.5% (vol/vol) level, the frequency of the Fe-CO band is $\sim 405\text{ cm}^{-1}$ which corresponds to a distal heme pocket conformation in which the imidazole side chain is in the pocket, as observed under most ambient conditions in the pH range from 6 to 8 [67]. When this same solution of COHbA is encapsulated in an RM at an added volume that matches that which is used to create a 20x sample for a glycerol free aqueous solvent, there appears a strong second peak at 492 cm^{-1} . This frequency corresponds to the “open” conformation typically seen at low pH where the imidazole is fully protonated. In this conformation, the protonated imidazole is rotated out of the distal heme pocket and stabilized by external waters that result in an open heme pocket. It is likely that the strong surface charge on the interior surface of the AOT RM's promotes this open configuration.

3.4. Reverse micelle incorporated Hb-hydration effects

Fig. 6 shows the HPT fluorescence spectra from 20x RM samples of free HPT plus added PEG, and HPT bound to HbA in the presence and absence of added PEG. The RM samples all have glycerol incorporated into the aqueous component (12.5% by volume with respect to the total volume of water plus glycerol). In the absence of PEG, both samples yield a spectrum that is essentially the single red shifted peak indicative of a population of mobile water surrounding the probe as seen in aqueous solution [20,40]. The immobile water shoulder at $\sim 450\text{ nm}$ is evident in the COHbA before the addition of PEG. The free probe also manifests this same shoulder in the absence of any osmolytes as was reported earlier [20]. The addition of PEG results in a noticeable increase in the blue shifted peak indicating the buildup of a population of immobile waters surrounding the probe for both the COHbA sample and the free probe. The several nanometer difference in the peak position for the blue peak when comparing the HPT and HPT + HbA samples is similar to what was reported earlier and can be taken as evidence that under these RM conditions the HPT probe is fully associated with the DPG binding site of HbA as discussed in our earlier work [20].

The bar graphs on the bottom of Fig. 6 show the relative change in the fraction of mobile water to total water (mobile plus immobile) as a function of added PEG (left bar graph) and 1.0 M urea (right bar graph). With added PEG there is a progressive increase in the relative amount of “immobile” water relative to mobile water (as seen in bulk phase water). This observation is consistent with the anticipated behavior that loss of water from the RM will increase the relative fraction of waters that are in close contact with the inner wall of the RM. These “inner wall” waters have been shown to be less mobile than the waters found in the interior central portion of the RM [28,68–75]. The urea result is shown to compare with earlier results that show that adding urea reverses the glycerol and trehalose-induced buildup of the immobile population waters and weakens the hydrogen bonding network in the aqueous solvent [19]. It can be seen that the same effect is observed for the RM.

Fig. 7 shows how the HPT plus HbA fluorescence spectrum changes in response to added osmolytes for a 10x RM suspension. The top panel shows the progressive buildup of the immobile water population as a function of added glycerol as was observed in solution and sol-gel samples. The bottom panel shows how the buildup of the immobile population due to the inclusion of trehalose can be enhanced with the addition of PEG and that the PEG

effect slowly builds with time, consistent with the hypothesis that the PEG effect is due to osmotic stress imposed on the RM from the external PEG.

The fluorescence from HbA labeled with Badan reports on the water environment surrounding the thiol coordinated Badan chromophore at the allosterically significant 93 Cys site. Fig. 8 shows how the peak position shifts for Badan fluorescence from Hb-Bd in both the RM (top panel) and the solution (bottom panel) as a function of added osmolytes. The solution phase sample shows minimal change with the added osmolytes whereas the RM sample shows progressive blue shifting with the addition of the osmolytes. The difference in the response between the two samples appears to originate from the initial impact of RM encapsulation on the local environment of the Badan probe. The peak position in the RM sample is initially red shifted compared to that of the solution phase sample, and with the addition of osmolytes, reaches the same end point as the solution phase sample. The red shift is consistent with a slight enhancement of water exposure for the protein bound probe [44]. This conclusion is supported by our observation (not shown) that for Hb-Bd in solution and in a sol-gel, the initial peak position shifts from 470 nm to 527 nm and 506 nm, respectively when denaturants are added (either 6 M urea or 5 M GdHCl). The confinement condition of the sol-gel has been shown to limit the degree of denaturant-induced “unfolding” [47,76,77]. The implication is that added osmolytes can be used to reverse the seemingly minor loosening effect of the RM on at least some aspects of conformation.

3.5. CO recombination

Fig. 9 shows the CO recombination traces for photodissociated COHbA in RMs under two conditions in comparison to COHbA encapsulated in a thin sol-gel film bathed in an excess of pure glycerol. The CO recombination trace initiated by a nanosecond photodissociation event can be broken down into two general processes: geminate recombination (GR) and solvent phase recombination (SR). Geminate recombination refers to the recombination of the dissociated CO with the initial parent heme from within the globin without having escaped into the surrounding solvent prior to rebinding. Solvent phase recombination refers to recombination involving the solvent derived ligand pool that includes ligands that have escaped into the solvent from the protein subsequent to photodissociation. The geminate recombination process can be further broken down into two subprocesses: GR1 which entails the initial geminate recombination process occurring from within the distal heme pocket prior to thermally driven diffusion of the CO to the various packing defect cavities (AKA Xe cavities) and the slower GR2 phase that occurs on a time scale where the dissociated CO is undergoing thermally facilitated diffusion among the accessible cavities within the globin. In low viscosity solvents at ambient temperature, nanosecond and slower CO recombination traces for HbA manifest primarily the GR2 and SR phases. The onset of the GR2 phase is subnanosecond under these conditions due to the subnanosecond onset of the influence of the side chain motions needed to facilitate diffusion out of the distal heme pocket. Under high viscosity or low temperature conditions, the transition from GR1 to GR2 can slow down to the point where it is easily observed on these time scales as shown for the glycerol bathed sol-gel samples shown in Fig. 9. The recombination trace for the glycerol bathed sol-gel sample shows how the increase in local viscosity due to the decrease in temperature essentially shuts down the process whereby both the CO escapes into the solvent and solvent based CO reenters the distal heme pocket [13]. The higher temperature sample shows the clear termination of the geminate phase followed by the solvent phase recombination (SR). At the lower temperature only the two geminate phases are observed. The recombination trace of the RM sample with the lower fraction of glycerol at the higher temperature contains a prominent solvent phase which matches that seen for the sol-gel sample and solution phase samples that reflect the solvent phase recombination for the R quaternary state of hemoglobin. Whereas the solution phase samples manifest a sizable

amplitude for the geminate phase associated with R state HbA, the amplitude for the geminate phase for the lower glycerol content RM sample is dramatically reduced. Reduction in the geminate yield of this magnitude is seen for Hbs stabilized in the T state; however, here the solvent phase is clearly indicative of an R state species. The low geminate yield for this sample is more likely due to the “open” configuration of the distal histidine which might allow for a very rapid escape of the CO into the solvent. It can be seen that lowering the temperature and increasing the glycerol content in the RM results in a dramatic change in the recombination trace. Now the recombination trace consists only of the single GR1 phase. This observation is indicative of a situation in which the local viscosity on the surface of the protein is increased to a level where the small side chain motions of the buried amino acids that are coupled/slaved to the motions of the hydration shell waters are highly damped. As a result, diffusion of the dissociated CO out of the distal heme pocket is greatly slowed compared to the recombination time from within the distal heme pocket-hence only GR1 is observed. This situation is usually apparent only at cryogenic temperatures well below 200 K [78,79].

4. Discussion

Incorporation of proteins into reverse micelles with retention of functionality and native conformation has been an ongoing research challenge with mixed success. The present work represents an advance with respect to this objective. Past efforts to incorporate hemoglobins and myoglobins have not been especially successful. The new protocol permits the preparation of reverse micelles that contain derivatives of HbA and Mb that exhibit spectroscopic properties indicative of native or near native conformations. An advantage of being able to prepare HbA or Mb containing reverse micelles is that these proteins are both well characterized with respect to function, conformation and dynamics and are well suited for a very wide range of optical probe techniques that address issues relating to the role of hydration and protein dynamics. The present study clearly shows: (i) how added PEG can be used to osmotically tune the water content of RM's loaded with either protein or probes; and (ii) how fluorescent probes that have been used effectively to characterize hydration properties in solution and other matrices can be used to follow hydration properties in the reverse micelles as a function of osmolyte additions and water content. The probes can be used to specifically target the hydration effects at well defined sites in proteins. HPT permits the direct targeting of the terminus of the water filled central cavity of HbA (AKA the DPG binding site) and Badan can be covalently attached to reactive thiols. In addition to the RM results obtained using the Badan labeled HbA, preliminary results (not shown) were also obtained both from a recombinant Mb with a cysteine introduced near the C terminus of the protein and a recombinant form of calmodulin where the site of Badan attachment reports on the apo-holo conformational changes [80].

Overall the results show that hydration properties in the reverse micelle can be tuned in a much more facile manner than in bulk solution. This feature should allow for systematic evaluation of how the hydration waters impact protein properties. The CO recombination traces from COHbA in the reverse micelles, although still in the exploratory stage, have implications with respect to how the reverse micelles can be used to test fundamental biophysical hypotheses relating to the interplay between hydration and protein dynamics.

Functionally important protein dynamics manifest a myriad of amplitudes and time scales. This inherent complexity makes the systematic study of both dynamics and their relationship to solvent motions challenging. The Solvent Slaving Model (SSM), proposed and developed by Frauenfelder and coworkers, groups protein dynamics based on the class of solvent motion through which they are activated [1–3]. An extension of the SSM, called The Protein Dynamic State Model (PDSM), defines dynamic states based on the temporal window

during which specific solvent slaved dynamics are active [7,13]. Together these two models provide a useful foundation for organizing, studying and analyzing protein dynamics.

The Solvent Slaving Model postulates that functionally important protein dynamics are activated by, and hence slaved to, the motions of the surrounding solvent. As a consequence, the vast array of different protein dynamics can be organized based on which category of solvent motion activates the specific observed protein dynamical process under a given set of conditions. This requirement for solvent activation as a prerequisite for specific protein motion is the principle that forms the basis for the idea that protein motions are slaved to the solvent motion. The major manifestation of this “slaving” is that the activation energy (E_a) of the protein motion is the same as that of the solvent dynamical process to which it is slaved. The underlying premise is that in the absence of solvent motion, the protein is inert, i.e. the activation (E_a) for the protein motion is very high. Two broad categories of solvent slaved protein dynamics have been identified: α and β slaved protein dynamics. The α and β slaved protein dynamics are those protein motions that manifest E_a 's that match those of the so-called α and β relaxations of the surrounding solvent, respectively.

The α relaxations that are linked to protein dynamics are those attributed to local non-translational motions of the solvent waters such as librational motions of hydration waters on the surface of the protein. These protein hydration shell α relaxations are not the same as those α relaxations occurring in the very different milieu of the bulk solvent and can therefore have a different E_a . The β slaved protein motions are anticipated to be small amplitude motions that do not necessarily require activation by large volume changing fluctuations on the part of the surrounding solvent. The larger amplitude α relaxations of the solvent involve translational motions related to dielectric relaxation properties. They are anticipated to be associated with protein dynamical processes that are facilitated by volume changing processes.

The time scale for a given observed protein dynamical process will depend upon: (i) the temperature and solvent dependent E_a 's for the α and β relaxations of the surrounding medium which determines the frequency of occurrence for each category of solvent fluctuation; and (ii) the number of solvent fluctuation steps required for either the α or β relaxations to complete the slaved protein dynamical process. Typically one can anticipate that the larger amplitude fluctuations of the solvent will be more effective as facilitators of protein motions, i.e. a given protein dynamical process will require fewer solvent fluctuation steps when slaved to α relaxations compared to when slaved to β relaxations. Thus if one initiates an observation over time starting at some time $t = 0$ and follows the time course of protein dynamical processes, the onset for observing α and β slaved motions will depend strongly on the relative E_a 's for the α and β relaxations as well as the number of α and β relaxation steps required to achieve the specific dynamical process. The E_a implies a time scale for the onset of influence of the different solvent fluctuations where the higher the E_a the slower the onset within a given observation time window. At low temperature and/or high viscosity the E_a for the α relaxations can greatly exceed those of the β relaxations, thus creating a clear cut initial temporal window where only β slaved processes are observed. The E_a 's for the β relaxations have a much steeper temperature and viscosity dependence compared to the E_a for α relaxations. As a consequence, the E_a 's for α relaxations drop substantially as the viscosity is lowered, and with increasing temperature their onset of influence in the temporal observation window for protein dynamics noticeably and dramatically shifts to shorter time. This assessment of the interplay between solvent and protein motions can be used to evaluate the implications of the CO recombination traces derived from the RM encapsulated HbA samples.

There are two major transitions/breaks in the kinetic traces associated with ligand recombination for hemeproteins that directly report on the onset of specific categories of protein motions. The first (with respect to temporal sequence starting at the moment of ligand-iron dissociation) is the break in the trace that separates the two geminate phases. It is connected to the activation/onset of small side chain motions that facilitate ligand diffusion among space filling cavities such as the distal heme pocket and the Xe cavities. This dynamical process, both because it is observed to persist even when the surrounding matrix has become glassy [81] and because it has a low E_a , can readily be assigned as a "slaved" process. The second transition terminates the observed geminate processes and initiates ligand escape and solvent phase recombination. It appears to be slaved to the "relaxations" because: (i) it is not observed in glassy matrices, (where the bulk waters are immobilized and "relaxations" are highly damped); (ii) exhibits dramatic temperature dependence above the glass transition; and (iii) has a high E_a . The results in the present study show that for a higher water content RM, the kinetic trace displays a solvent phase consistent with "relaxations" still being active. What is unexpected is that for the lower water content RM sample at a slightly lower temperature, not only is the second transition gone but so is the first transition that separates the two geminate phases. It can be seen that for a sol-gel encapsulated sample bathed in pure glycerol, the temperature drop is sufficient to eliminate the purported "slaved" second transition but the first transition between the two geminate phases is still observed. Indeed it is still observed even at temperatures well below the glass transition for trehalose glasses as well as for glycerol bathed sol-gel samples [26] [47]. The loss of the first transition does occur at very low cryogenic temperatures but is also reported to occur in ultra-dry glasses at ambient temperatures [82]. The implication is that in the low water content RM, the "slaved" dynamics that encompass the side chain motions needed to facilitate CO diffusion, are not active on the time scale of the observed geminate recombination. This observation might be viewed as an indication that protein motion is completely frozen; however, the line shape of the observed GR1 process for the low water content RM appears to be exponential in contrast to the cryogenic results where the line shape is highly non-exponential indicative of a frozen distribution of enthalpic barriers controlling rebinding [13]. The present finding suggests that although the side chain mediated diffusive processes are "frozen", conformational averaging at the iron is still operational. This possibility is supported by our finding that the E_a for a dynamic hole filling process that can be associated with conformational averaging at the iron has an E_a of only several kJ/mol whereas the E_a for the slower side chain motions associated with the GR1 to GR2 transition is on the order of 15–30 kJ/mol (unpublished results). The presented results and the analysis are, of course, limited but indicate that RM encapsulation can allow for extreme tuning of hydration driven process in proteins. This potential benefit along with the ability to precisely control and monitor the water content and water properties within the RM should permit more detailed evaluation of models accounting for the role of water and protein functionality. Most significant is the potential benefit of using the Hb in the RM system as an appropriate molecular laboratory for: (i) comparing the RM derived hydration effects with those obtained from other confinement matrices such as glasses and sol-gels; and (ii) establishing a more comprehensive macro/micro level picture of how hydration impacts the different tiers of protein dynamics by dissecting dynamics and hydration properties using the same RM system with both optical and NMR probes.

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Fig. 1.

(A) Fluorescence emission spectra of HPT (10 μM) in a 0.1 M AOT/isooctane reverse micelle ω_O 10X: — 0.05 M Bis-Tris, pH 6.5; — buffer + 25% glycerol; — buffer + 50% glycerol; — buffer + 75% glycerol. Ex. 355 nm. (B) Fluorescence emission spectra of HPT (10 μM) in a 0.10 M AOT/isooctane reverse micelle ω_O 10X: — 0.05 M Bis-Tris pH 6.5 buffer; — buffer 30 min post solid PEG added to the reverse micelle solution; — same solution after shaking. Ex. 355 nm. (C) Fluorescence emission spectra of HPT (10 μM) encapsulated in a trehalose glassy matrix with 0.05 M Bis-Tris, pH 6.5 buffer and 0.5 M cation salts: — HPT in buffer; — HPT in buffer + Na^{+1} ; — buffer + Li^{+1} ; — buffer + Mg^{+2} . Ex. 355 nm.

Fig. 2.

(A) Fluorescence emission spectra of GSH-BD in solution; — 0.05 M Bis-Tris pH 7.0; — buffer + 0.5 M trehalose; — buffer + 50% glycerol; — buffer + 50% glycerol + trehalose. Ex. 385 nm. (B) Fluorescence emission spectra of GSH complexed to Badan encapsulated in a trehalose — or a sucrose — glassy matrix. Samples were dried and heated for 1 h at 40 °C. Ex. 385 nm.

Fig. 3.

(A) Fluorescence emission spectra of GSH complexed to Badan encapsulated in a 0.10 M AOT/isooctane reverse micelle as a function of water:AOT ratio ω_0 — 5X; — 10X — 20X. Ex. 385 nm. (B) Fluorescence emission spectra of GSH-BD in 0.05 MBis-Tris, pH 7.0 encapsulated in a 0.10 MAOT/isooctane reverse micelle with 0.5 M Trehalose; — 50% glycerol; — 50% glycerol + trehalose, ω_0 10X. Ex. 385 nm.

Fig. 4. Absorption spectrum of COHbA modified with Aedans (1:1 heme) encapsulated in a 0.1 M AOT/isooctane reverse micelle; 0.05 M Bis-Tris, pH 7.0 + 25% glycerol $\times 20X$.

Fig. 5.

(A) Circular Dichroism spectrum of COHbA encapsulated in a 0.10 M AOT/isooctane reverse micelle, ω_O is 10X: 0.05 M pH 7.0, 50% glycerol, 1 mm cell demonstrating the helical content ; protein concentration is 30 μ M. (B) The same sample of COHbA in the far UV region of the CD spectrum; protein concentration is 60 μ M.

Fig. 6. (A) Fluorescence emission spectrum of COHbA complexed with HPT 1:1 heme, encapsulated in a 0.10 M AOT/isooctane reverse micelle; ω_0 is 20X: — 0.05 M Bis-Tris, pH 6.5 + 12.5% glycerol; — COHbA – HPT complex after the addition of solid PEG 3 K to the reverse micelle solution; — HPT solution encapsulated in a 0.10 M AOT/isooctane reverse micelle after PEG 3 k is added to the solution; HPT is 10 μ M in 0.05 Bis-Tris, pH 6.5; ω_0 is 20X. Ex. 355 nm. (B) Graph representing the ratio of the red emission peak of HPT to the blue emission peak as a function of osmolyte added to the reverse micelle; conditions as in A. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 7.

(A) Fluorescence emission spectrum of COHbA complexed with HPT 1:1 heme, encapsulated in a 0.10 M AOT/isooctane reverse micelle ω_O is 10X; — 0.05 M Bis-Tris, pH 6.5 + 25% glycerol; — buffer + 50% glycerol; — buffer + 75% glycerol. Ex. 355 nm. (B) Fluorescence emission spectrum of COHbA complexed with HPT 1:1 heme encapsulated in a 0.10 M AOT/isooctane reverse micelle ω_O is 10X; — 0.05 M Bis-Tris, pH 6.5 + 0.5 M trehalose; — buffer + trehalose + PEG 3 K; — same sample 13 days post PEG 3 K. Ex. 355 nm.

Fig. 8.

(A) Fluorescence emission spectra of Hb – Badan encapsulated in a 0.10 M AOT/isooctane reverse micelle ω_0 10X: — 0.05 M Bis-Tris, pH 7.0; — Hb-Badan in buffer + 50% glycerol; — Hb-Badan in buffer + 50% glycerol + 0.5 M trehalose. Emission maxima are noted. Ex. 385 nm. (B) Fluorescence emission spectra of Hb – Badan — in buffer solution; — in buffer solution + 50% glycerol; — in buffer solution + 50% glycerol + 0.5 M trehalose; Ex. 385 nm. Emission maxima are noted. Ex. 385 nm.

Fig. 9.

The CO recombination traces for photodissociated COHbA at 3.5 °C in 0.1 M AOT/ isooctane reverse micelle 10X: — in 0.05 MBis-Tris pH 7.0 + 12% glycerol; — in buffer + 50% glycerol; sol-gel encapsulated HbCO in 100% glycerol — at 25°C and at — 3.5°C.