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Probing Water Density and Dynamics in the Chaperonin GroEL Cavity

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Supporting Information

ABSTRACT: ATP-dependent binding of the chaperonin GroEL to its cofactor GroES forms a cavity in which encapsulated substrate proteins can fold in isolation from bulk solution. It has been suggested that folding in the cavity may differ from that in bulk solution owing to steric confinement, interactions with the cavity walls, and differences between the properties of cavity-confined and bulk water. However, experimental data regarding the cavity-confined water are lacking. Here, we report measurements of water density and diffusion dynamics in the vicinity of a spin label attached to a cysteine in the Tyr71 → Cys GroES mutant obtained using two magnetic resonance techniques: electron-spin echo envelope modulation and Overhauser dynamic nuclear polarization. Residue 71 in GroES is fully exposed to bulk water in free GroES and to confined water within the cavity of the GroEL−GroES complex. Our data show that water density and translational dynamics in the vicinity of the label do not change upon complex formation, thus indicating that bulk water-exposed and cavity-confined GroES surface water share similar properties. Interestingly, the diffusion dynamics of water near the GroES surface are found to be unusually fast relative to other protein surfaces studied. The implications of these findings for chaperonin-assisted folding mechanisms are discussed.

INTRODUCTION

The Escherichia coli GroE chaperonin system facilitates protein folding in vivo and in vitro in an ATP-dependent manner (for reviews see, for example, refs 1–3). It comprises GroEL, an oligomer of 14 identical subunits that form two heptameric rings, stacked back-to-back, with a cavity at each end in which protein folding can take place in a protective environment, and its helper-protein GroES, which is a homoheptameric single ring. The GroE system is essential for the folding of only a small subset of E. coli proteins (<100) but what distinguishes GroE clients from all other E. coli proteins remains unclear.4 Obligate substrates or other non-native proteins can become encapsulated in the GroEL cavity when GroES binds to the apical domains of a substrate- and ATP-occupied GroEL ring. The substrates are then discharged into bulk solution, either folded or not, following GroES dissociation that is triggered by ATP hydrolysis in the GroES-bound cis GroEL ring and ATP binding to the opposite trans GroEL ring (see refs 2 and 7 for detailed schemes of current models of the GroE reaction cycle). The reaction cycle of GroEL is governed by the cooperative binding of ATP that is positive within rings and negative between rings.5 The intraring positive allosterie facilitates cycling of the GroEL rings between protein substrate acceptor and release states. Inter-ring negative allosterie ensures that the two rings can operate out-of-phase with respect to each other and that ATP binding to one ring triggers GroES release from the opposite ring.5–7 However, the role of the inter-ring allosterie is less clear when the symmetric "football-shaped" GroEL−GroES2 complex (and not the asymmetric GroEL−GroES complex) is the active species of this nanomachine.6

Despite more than two decades of intensive research, it remains unclear and controversial whether the cavity of the GroEL−GroES complex is only a “passive cage” in which aggregation is prevented or whether the folding pathway is unchanged8 or a chamber that has evolved to optimize the folding process itself.9,10 Factors that could influence the folding reaction inside the GroEL cavity are steric confinement,9,10 the chemical nature of the cavity walls,9,10 and the properties of the cavity-confined water, which may, in fact, be intimately linked to the steric and/or chemical effects of the confinement imposed by the GroEL interior surface.11 The extent of steric confinement and the chemical nature of the cavity walls are known from the crystal structure of the GroEL−GroES complex,6 but there is no available experimental data regarding the properties of the cavity water. Specifically, insight into the diffusion dynamics of water within the GroEL cavity can offer critical clues about the GroEL surface water attraction and may allow us to hypothesize about the stability and folding potential of proteins entering the GroEL cavity. If water is interacting favorably with the interior surface of the GroEL cavity, as would be reflected in strongly retarded, rigidified, surface water dynamics,12 then a protein substrate that is encapsulated in the cavity will experience a strongly repulsive hydration barrier from the GroEL surface.
and, thus, tend to fold in order to bury its hydrophobic residues.\textsuperscript{11} By contrast, the hydrophobic nature of the cavity walls in GroEL’s substrate acceptor state\textsuperscript{6,6} may be reflected in nonretarded, fast diffusing, surface water dynamics that disfavor substrate folding. Equally interesting is the surface of the GroES lid: is it strongly or weakly hydrated and do the hydration level and dynamics change upon formation of the GroEL—GroES complex? The hydration properties of the GroEL cavity have been the focus of computational studies that indicated, for example, that GroEL’s ability to assist folding scales with the affinity for water of the cavity’s interior surface.\textsuperscript{11} However, direct experimental measurements of properties of confined water in the GroEL cavity have not yet been reported. In this study, we present the first such experimental measurements for water near the surface of free GroES and the same surface when it faces the cavity of the GroEL—GroES complex.

The experiments described here combine site-directed spin labeling (SDSL) with two state-of-the-art magnetic resonance techniques: electron-spin echo envelope modulation (ESEEM) and Overhauser dynamic nuclear polarization—enhanced nuclear magnetic resonance (ODNP-NMR). A single site, Tyr71, in GroES was replaced by site-directed mutagenesis with a cysteine to which a nitroxide spin label, N-(1-oxyl-2,2,5,5-tetramethyl pyrroldiny1l)-maleimide, was attached. This position was chosen since it is fully exposed to bulk water in unbound GroES and, upon GroEL—GroES complex formation, faces the confined water inside the chaperonin cavity (Figure 1A,B).

Importantly, the spin label at this position is sufficiently far-removed from residues in the cavity wall, with the closest residue being Asn299, whose Cβ side-chain atom is about 17 Å away from the nitroxide oxygen. The single-ring (SR1) version of GroEL with the mutation Asp398 → Ala that slows ATP hydrolysis considerably\textsuperscript{13} was studied here instead of wild-type GroEL in order to minimize dissociation of the labeled GroES from GroEL. The cavity properties and intraring allostery\textsuperscript{14,15} of SR1 are similar to those of wild-type GroEL.

ESEEM and ODNP-NMR spectroscopy at X-band (\textasciitilde10 GHz) frequencies and a magnetic field of 0.35 T were employed to probe the properties of local water within the chaperonin cavity. In order to probe the amount of water in the vicinity of the spin label that protrudes into the cavity of the GroES—GroEL complex and can sense its upper region, the well-established ESEEM technique was employed for measuring hyperfine interactions between the electron spin of the label and nearby nuclear spins.\textsuperscript{16} When the hyperfine interaction is very weak, its isotropic part is zero and the anisotropic part can be described by the point dipole interaction between the electron spin and the nuclear spin, whose strength is inversely proportional to the cube of their distance, \( r \). In such cases, this interaction is manifested as modulations in the electron spin echo decay that oscillate at a frequency equal to the Larmor frequencies of the coupled nuclei, and the number of weakly coupled magnetic nuclei and their average distances from the electron spin are reflected in the modulation depth. By combining ESEEM of \( ^1\text{H} \) nuclei in D\textsubscript{2}O solutions with spin labeling, it is possible to probe the number of D\textsubscript{2}O molecules in the vicinity of the spin-labeled residue Cys71 (up to about 8 Å) without interferences from the protein protons. This method has been successfully used to derive the water penetration depth in membranes\textsuperscript{17,18} and water exposure of protein residues.\textsuperscript{19,20}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Figure_1.png}
\caption{(A) Side and (B) top views of single-ring GroEL in complex with spin-labeled GroES. GroEL and GroES in the crystal structure of the GroEL—GroES complex (PDB code: 1AON)\textsuperscript{6} are represented by space-filling (in gray) and ball-and-stick (in magenta) models, respectively. The labeled GroES subunit is shown in a darker magenta. The sulfur, carbon, nitrogen, and oxygen atoms of the spin label are shown in orange, yellow, blue, and red, respectively. In panel A, the apical, intermediate and equatorial domains are designated by \( a, b, \) and \( c \), respectively, and two subunits of GroES were removed in order to reveal the cavity. In the single-ring GroEL—GroES complex, the spin-labels are exposed to confined water in the cavity and are not close to any residues of GroEL. The figure was generated using the Chimera software.\textsuperscript{51}}
\end{figure}

ESEEM measurements are usually carried out in frozen solutions and cannot probe the dynamic properties of protein surface water. To get information regarding dynamics under solution conditions at room temperature, we applied ODNP-NMR relaxometry\textsuperscript{21,22} to probe the diffusion dynamics of water near the spin-labeled Cys71. ODNP selectively amplifies the \( ^1\text{H} \) NMR signal of the local hydration water around a specific spin label (within 5–10 Å) of a protein site by transferring polarization from the electron spin to the nearby moving water molecules using the same anisotropic hyperfine interaction mentioned above (alternatively termed the electron-nuclear dipole–dipole interaction). ODNP relies on the enhancement of the \( ^1\text{H} \) NMR signal of water at 0.35 T and \textasciitilde15 MHz that is achieved by saturating the electron spin resonance (ESR) transitions at \textasciitilde10 GHz. Since only the \( ^1\text{H} \) of water molecules that move fast (relative to \textasciitilde10 GHz) experience electron–\( ^1\text{H} \) spin flip-flops that give rise to \( ^1\text{H} \) NMR signal enhancement, ODNP can be exploited to quantify local water diffusivity near the nitroxide spin label. The motion of hydration water is
characterized by a translational diffusion correlation time ($\tau_c$), which represents the time needed for water to diffuse near the spin label within a distance $b$ (typically $5-10$ Å, as determined by the electron-1H dipolar coupling field) and is inversely proportional to the local diffusion coefficient ($D$), i.e., with $\tau_c \propto b^2/D$. Crucially, ODNP, when combined with $^1$H NMR relaxation time measurements, can separate contributions of freely diffusively translating hydration water ($k_0$ picosecond time scale) from motional fluctuations that occur on a slower time scale ($k_0$ nanosecond time scale).22 Weak protein surface water attraction will be reflected in small $\tau_c$ and large $D$ and large $k_0$ values. Strong protein surface water attraction will present the opposite trend of large $\tau_c$ and small $D$ and small $k_0$ values. In addition, there can be contributions from strongly bound water on protein surfaces with lifetimes exceeding $\sim 1$ ns, whose presence would be reflected in a large $k_0$ value that increases as the rotational tumbling of the protein is slowed, for example, upon immobilization or immersion in a viscous solvent. Using this approach, the hydration dynamics landscape of dihydrophobic lipid membrane surfaces32 or representative protein surfaces.33 The ESR and ESEEM experiments were carried out using a Bruker ELEXSY E580 spectrometer (9.5 GHz) using an ER4118X-MS-5 probe-head with a split ring resonator (5 mm sample access) on a Bruker ELEXSY E500 spectrometer, using round quartz capillaries measured routinely using gel-filtration chromatography. The ODNP experiments were carried out using 240 $\mu$M labeled or unlabeled GroES and a 1.5 molar excess of SR1 in G10K buffer containing 4 mM ATP and 21% (w/v) Picoll 70 where indicated.

**MATERIALS AND METHODS**

**Molecular Biology.** The gene coding for GroES fused to a His$_6$-tag at its C-terminus and containing the Tyr71 $\rightarrow$ Cys mutation was generated using the plasmid pOAA$^5$ and the Quick-Change site-directed mutagenesis kit (Stratagene, La Jolla, CA). The His$_6$-tag was introduced in two steps using the forward (and corresponding back) primers: His-tag 1,5'-CGTATTATTTTCAAGGATGATGGCTGCGGTGGT-GAAATCTGAGAAGATGC-3' and the corresponding back primer. DNA sequencing of the entire GroES gene was carried out to verify that the desired construct was obtained.

**Protein Purification.** GroES was purified by growing E. coli TG1 cells bearing the plasmid described above overnight at 37 °C in 2xTY medium containing 50 $\mu$g/mL ampicillin. The overnight culture was diluted 1:100 in 2xTY medium containing 50 $\mu$g/mL ampicillin, grown overnight at 37 °C and harvested. The pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.5) containing $\sim 0.10%$ (w/v) sucrose, centrifuged, and stored at $\sim -80$ °C until further use. It was then resuspended in 50 mM Tris-HCl buffer (pH 7.5) containing 0.5 M NaCl, 10 mM $\beta$-mercaptoethanol, 10 mM imidazole (buffer A), and 1 mM phenylmethanesulphonylfluoride. The cells were disrupted by sonication and the lysate was clarified by centrifugation at 20,000 rpm for 30 min at 4 °C. The supernatant was loaded on a 5 mL HisTrap HP column (Amersham Pharmacia, Uppsala, Sweden), and GroES was eluted using a 10--500 mM imidazole gradient in buffer A. Fractions were analyzed by SDS-PAGE and those containing GroES were combined and concentrated using a Vivaspin device (Sartorius, Goettingen, Germany) with a 10 kDa cutoff filter. The concentrated protein was transferred into 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM KCl and 10 mM MgCl$_2$ (G10K buffer) using a PD-10 desalting column (GE Healthcare, Uppsala, Sweden) and then concentrated again. Aliquots of protein were snap frozen in liquid nitrogen and stored at $\sim -80$ °C.

Purification of SR1, a single-ring version of GroEL, with the Asp398 $\rightarrow$ Ala mutation was carried out as described previously.24

**Spin Labeling of GroES.** A 50-fold molar excess of the 3-maleimido-2,2,5,5-tetramethyl-1-pyrroldinylxoy (3-maleimido-proxyl) spin probe (Sigma) was added to the GroES Tyr71 $\rightarrow$ Cys mutant in D$_2$O G10K buffer and the suspension was then shaken for 16 h at 37 °C. Under these conditions, complete labeling is assumed to occur. Excess spin label was separated from the labeled GroES by using MicroSpin G-25 buffer exchange columns (GE Healthcare, Uppsala, Sweden). The labeled GroES was divided into aliquots, snap-frozen in liquid nitrogen and stored at $\sim -80$ °C. ESEEM and ODNP experiments were not carried out using the more standard 5-(2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrrol-3-yl)methylmethanesulphonothioate (MTSL) label since GroES in complex with GroEL loses this label over time for reasons that are not clear.

**Sample Preparations.** The ESR and ESEEM experiments were carried out using an SR1-GroES complex that was prepared by incubating 1 mM ATP with 12 $\mu$M SR1 for 30 s and then adding labeled GroES (all in D$_2$O G10K buffer) and incubating for an additional 5 min. The molar ratio between SR1 and labeled GroES was 1:5, respectively, in order to ensure that all the labeled GroES is GroES bound. This was verified routinely using gel-filtration chromatography. The ODNP experiments were carried out using 240 $\mu$M labeled or unlabeled GroES and a 1.5 molar excess of SR1 in G10K buffer containing 4 mM ATP and 21% (w/v) Picoll 70 where indicated.

**ESR and ESEEM Measurements.** All CW X-band (9.5 GHz) measurements were performed at room temperature (22–25 °C) on a Bruker ELEXSY E500 spectrometer, using round quartz capillaries (0.075 mm i.d. and 1 mm o.d.). ESEEM experiments were carried out at 80 K on a Bruker ELEXSY E580 spectrometer (9.5 GHz) using an ER4118X-MS-5 probe-head with a split ring resonator (5 mm sample access) on $\sim 50$–$60$ $\mu$L sample volumes. The ESEEM experiments were carried out using the three-pulse sequence $\pi/2-\tau-\pi-\tau-\pi/2-\tau$, with a repetition time of 2.5 ms and a four-step phase cycling, in the presence of a magnetic field set to maximum echo intensity.25 The $\pi/2$ pulse length was 16 ns. The $\tau$ value was optimized to maximize the modulation depth of $^1H$, i.e., $\tau = \tau_{c}/(2\nu_{H})$ while minimizing the modulation depth of $^2H$, i.e., $\tau = \tau_{c}/(4\nu_{H})$, yielding $\tau = 208$ ns, where $\nu_{H}$ is $^1H$ or $^2H$ Larmor frequency, respectively. The time interval $T$ was incremented in 20 ns steps starting at 60 ns for a total number of 250 points. The ESEEM modulation was isolated from the signal trace and its Fourier transform (FT-SEEM) as follows: (1) phase forward primer: 5’-CGTATTATTTTCAAGGATGATGGCTGCGGTGGT-GAAATCTGAGAAGATGC-3’ and the corresponding back primer. DNA sequencing of the entire GroES gene was carried out to verify that the desired construct was obtained.
The presence of spin labels has two effects: (i) with or without microwave irradiation, the spin labels lead to a faster NMR relaxation rate, $R_1$ (Figure 2A,B); and (ii) in the presence of saturating microwaves, the ESR transition will cross-relax with the NMR transition of the $^1$H nuclei of water (at a rate given by $k_sC_{SL}$ as described below), thereby leading to an enhanced $^1$H NMR signal (Figure 2C). These two effects were quantified by carrying out NMR inversion recovery experiments (Figure 2A,B) and a series of basic NMR free induction decay (FID) experiments (Figure 2C) over a range of microwave powers. In both cases, the resulting NMR signals were Fourier transformed, baseline corrected, and integrated (pulse sequences in Figure 2A–C yield the respective data in Figure 2D–F). The integrated FT NMR signal from the FID experiments (Figure 2C) was normalized against the signal in the absence of microwave power to illustrate the increasingly larger enhancements (i.e., $E(p)$) obtained with increasing powers of saturating microwaves (Figure 2F). The inversion recovery data (Figure 2F) reflects the rate of recovery of the nuclear magnetization from the inverted state to equilibrium (i.e., $R_1(p)$). Finally, a control measurement is performed on a sample prepared without spin label. This consists of an inversion recovery experiment in the absence of microwave power (Figure 2A), which reflects the rate of recovery of magnetization to equilibrium in the absence of spin label, $R_{1,0}$ (note that here $R_1$ and $R_{1,0}$ refer to the inverses of the NMR spin–lattice relaxation times, i.e. $R_1 = T_1^{-1}$ and $R_{1,0} = T_{1,0}^{-1}$).

The data shown in Figure 2D–F were further processed to obtain the spin label-dependent relaxation rates, or relaxivities, that offer insight into the dynamics of the hydration water, as explained in more detail elsewhere. The inversion recovery curves (e.g., Figure 2D, E) are fitted to obtain the NMR relaxation rates of samples without the spin label ($R_{1,0}$, Figure 2G) and with the spin label ($R_1(p = 0)$ from Figure 2H). The self-relaxation rate, $k_sC_{SL}$, is obtained by subtracting $R_{1,0}$ from $R_1(p = 0)$ (i.e., $R_1$ in the absence of microwave power). The

Figure 2. Outline of the complete procedure for ODNP data processing is shown for representative data. First, a variety of NMR measurements is carried out including an inversion recovery sequence acquired on a sample without spin label (A), a series of inversion recovery sequences acquired with spin label and different microwave powers (B), and a simple NMR spectrum acquired at different powers of ESR-resonant microwaves (C). The data corresponding to these pulse sequences are shown in panels D–F. The inversion–recovery curves (D, E) are fitted to determine the NMR relaxation rates $R_{1,0}$ (G) and $R_1(p)$ (H). The latter multiplies $1 - E(p)$ (F) to yield $k_s(p)$ (I), which are fitted to an asymptotic curve (shown as a solid line), allowing us to extrapolate it to full saturation of the ESR transition and determine $k_s \approx k_{s,max}$. The multiple curves in panels F, H and I are for repeated experiments as indicated by the color code in panel G.
spin-label-driven proton self-relaxivity, $k_{\rho}$, is then obtained from the self-relaxation rate by normalizing against the spin label concentration ($C_{SL}$). The cross-relaxivity, $k_{C}$, is determined from the data in Figure 2I, which are obtained by multiplying $1 - E(p)$, the amount of polarization transferred (Figure 2F), by the microwave power-dependent relaxation rate $R(p)$ (Figure 2H) and dividing by 659.3 (the ratio of the ESR to NMR resonance frequencies) and the concentration $C_{SL}$. These data are then fitted to an asymptotic curve to obtain a value for the cross relaxivity, $k_{C} \approx k_{cross} \approx 1$, as shown previously$^{41}$ where the value of $k_{C}(p)$ approaches complete saturation of the ESR transition at high microwave power.

The ratio of the relaxivities $k_{C}$ and $k_{\rho}$ yields the coupling factor, $\xi$ ($\xi = k_{C}/k_{\rho}$). Given a specific field (and therefore resonance frequency), the force-free hard-sphere (FFHS) model for translational dynamics$^{42}$ provides a relationship that can be used to determine the translational correlation time, $\tau_{c}$, from the measured value of $\xi$. In order to better understand the contribution of partially bound waters (which are not well modeled by FFHS) to the value of $\xi$, the contribution from the fast waters (i.e., $k_{n}$) can also be subtracted from the self-relaxivities ($k_{s}$) as follows:

$$k_{low} = \frac{5}{3} k_{s} - \frac{7}{3} k_{n}$$

(1)

where $k_{low}$ describes the slower time scale (~15 MHz) fluctuations of the dipolar interaction.$^{23}$ The value of $\xi$ is related to the ratio between $k_{s}$ and $k_{low}$:

$$\xi = \frac{5k_{s}/k_{low}}{3 + 7k_{s}/k_{low}}$$

(2)

where $0 \leq k_{s}/k_{low} \leq 1$. Each measurement was repeated 2–4 times, and the standard deviations of the resulting values of $\xi$, $k_{s}$, $k_{low}$, and $\tau_{c}$ are presented as errors (i.e., as value ± error). An analysis of the scatter in the data is shown in Figure S1.

## RESULTS AND DISCUSSION

**ESR Measurements.** The X-band ESR spectrum of the spin labeled GroES (SL-GroES) in Figure 3 shows that the mobility of the spin label at position 71 on the GroES surface (Figure 1) is restricted compared to a free spin probe and represents a single population, thus providing evidence that the spin label is attached to the protein. An estimate of 10$^{-9}$ s for the rotational correlation time can be obtained from comparison to spectra simulated using EasySpin$^{43}$ and assuming isotropic motion. Notably, the ESR spectrum shows only very subtle broadening upon formation of the complex between SL-GroES and SR1, thus indicating that the mobility of the spin label hardly changes when it is encapsulated within the cavity.

The ESEEM results for SL-GroES and the SL-GroES–SR1 complex in D$_{2}$O solvent are presented in Figure 4. The peak at the $^2$H frequency with intensity $I(2H)$ shows two components, where the broad resonance is due to water molecules H-bonded to the nitroxide moiety and the narrow component, $I(2H)_{narrow}$ is due to more distant water molecules.$^{23}$ The time domain and FT-ESEEM traces for the SL-GroES and SL-GroES–SR1 complex samples are identical, thus indicating that there is no difference between the density of water near the spin label of free SL-GroES vs SL-GroES in complex with SR1. This implies that the number of water molecules and their distances from the spin label are the same in the two samples as reflected in the same $I(2H) = 42$. For comparison, we also measured the $I(2H)$ value for a free spin label dissolved in D$_{2}$O/glycerol-d$_{8}$ (7:3 v/v) and obtained $I(2H) = 80$. Here, the addition of the glycerol was essential to prevent ice formation and aggregation of the spin probe upon freezing. A ratio of 0.5 is found between the $I(2H)$ values for the SL-GroES by itself or in complex with SR1 and the free spin label. Assuming that glycerol-d$_{8}$ does not affect significantly the $^2$H density in the sample (as glycerol was not present in the protein samples) and in the vicinity of the spin label, we can compare this value to the values of 0.54 and 0.18 that were obtained for the most exposed and buried MTSL-labeled sites, respectively, in the light harvesting protein complex IIIb of photosystem II.$^{20}$ This is consistent with the spin label attached to GroES being exposed to bulk or the cavity water. Currently, there is no reliable theoretical model for extracting the actual water distribution in the vicinity of the spin probe, in the case of D$_{2}$O solutions, from fitting the experimental data. Consequently, the data are often fitted to a model based on assuming a spherical distribution of $n$ $^2$H nuclei around the spin label at an effective distance $r$.$^{14}$ We chose not to use such a model as it is not realistic and preferred, instead,
to interpret the experimental $I(\tau)$ values on a comparative basis.

**ODNP Measurements.** Representative $R_c$ and $R_0^c$ data, as well as all the original $^1$H NMR signal enhancement measurements as a function of microwave power, $E(p)$, are shown in Figure 2. These data were collected for three samples: free GroES in G10K buffer, GroES in complex with SR1, and GroES in a Ficoll 70 solution. From these data, $k_c \approx k_{c\text{max}}$ values were extracted, as well as the $k_{c\text{max}}$ values using eq 1, the coupling factor, $\xi$, and the translational diffusion correlation time, $\tau_c$ (see Table 1). The ratios between the $k_c$, $k_{c\text{max}}$, $\xi$, and $\tau_c$ are given.

| Table 1. Relaxivity, Coupling Factor, and Retardation Factor Values for GroES under Different Conditions$^a$ |
|-------------------------------------------------|
| $k_c/k_{c\text{bulk}}$ | $k_{c\text{max}}/k_{c\text{max, bulk}}$ | $\xi/\tau_{c,\text{bulk}}$ | $\tau_c/\tau_{c,\text{bulk}}$ |
| GroES–SR1 complex | 0.36 ± 0.06 | 1.22 ± 0.24 | 0.40 ± 0.10 | 3.14 ± 0.72 |
| GroES | 0.34 ± 0.08 | 0.80 ± 0.35 | 0.55 ± 0.13 | 2.31 ± 0.55 |
| GroES with Ficoll 70 | 0.40 ± 0.06 | 0.64 ± 0.20 | 0.73 ± 0.26 | 1.64 ± 0.75 |

$^a$For derivation of the relaxivity values, see the text and Figure 2.

values for the spin label tethered to GroES and the free spin label in bulk solution are presented in Table 1 and Figure 5. It can be seen that the values of these ratios are the same, within error, for SL-GroES and the SL-GroES–SR1 complex. Therefore, we will first discuss the meaning of the resulting average values and the fact that the value of $k_c$ remains completely unaltered—the key result presented here. The meaning of very small changes in $k_{c\text{max}}/k_{c\text{max, bulk}}$ that impact the value of $\xi$ and $\tau_c$ (eq 2) will be discussed below. Interestingly, the value of $k_{c\text{max}}/k_{c\text{max, bulk}}$ that represents the contribution from slow time scale fluctuations is approximately 1, thereby indicating that it is likely that there is no bound water at the SL-GroES surface. This, by itself, is an interesting result as it is typical to find some contribution from bound water near protein surfaces, unlike at the surfaces of lipid membranes that are known to have minimal or no contribution from bound water. All the $k_{c\text{max}}/k_{c\text{max, bulk}}$ values are $0.4 \pm 0.07$ and, thus, reflect modest retardation and comparatively fast diffusive motion of the surface water hydrating the SL-GroES surface. These data clearly illustrate that the decrease in the $\xi$ values relative to those of bulk water and the retardation of surface water dynamics as reflected in $\tau_c/\tau_{c,\text{bulk}}$ originate exclusively from changes in the contribution of fast moving, loosely bound, surface water, as reflected in $k_c$. Moreover, the calculated value of $2–3$ for the retardation factor, $\tau_c/\tau_{c,\text{bulk}}$, is exceptionally small compared to typical retardation factors of $5–10$ or larger, as found for solvent-exposed protein surfaces of tau, apomyoglobin and other biomolecular or polymer surfaces25,32 (Figure 6). All of these trends point to a highly lubricated, weakly hydrated, protein surface of SL-GroES. This weak hydration does not change, within error, upon complexation with SR1. To further test this conclusion, the measurements of water dynamics were repeated for SL-GroES in the presence of 21% (w/v) Ficoll 70, a known viscoelastic that does not interact

Figure 5. Bar plot of the values of the various ODNP measurements for free GroES in aqueous buffer, GroES in complex with SR1 and GroES in the presence of Ficoll 70. Shown are values of the cross-relaxivity, $k_c$ (blue), the slow-motion component of the self-relaxivity,$^{22} k_{c\text{max}} = 5/3k_p - 7/3k_c$ (green), the coupling factor, $\xi$ (red), and the translational correlation time, $\tau_c$ (cyan), which is determined by applying the FFHS model. For simplicity, all quantities are normalized by the appropriate bulk values: $^c k_{c\text{max}} = 95.4$ s$^{-1}$ M$^{-1}$, $k_{c\text{max, bulk}} = 366$ s$^{-1}$ M$^{-1}$, $\xi,\text{bulk} = 0.27$, $k_p = 353$ s$^{-1}$ M$^{-1}$, and $\tau_{c,\text{bulk}} = 54$ ps.

Figure 6. Plot of the coupling factor measurement, $\xi$, as a function of the modeled translational correlation time, $\tau_c$. The data points for the ODNP measurements for free GroES, GroES in complex with SR1, and GroES in the presence of Ficoll 70 are in brown, red, and green, respectively. The FFHS model gives a fixed relationship, $\xi(\tau_c)$, for measurements at 0.35 T (corresponding to 15 MHz nuclear Larmor frequency) that is illustrated by the solid gray line. The gray symbols indicate previous ODNP measurements for a variety of proteins, small peptides, lipids, and DNA that are grouped (in brown text, to the right) according to the location of the spin label. As explained previously,$^{22}$ measurements in the zone designated “buried” were for labels attached within the core of a lipid bilayer, globular protein, or compact polymer system; in the zone designated “surface” for labels attached to the surfaces of proteins or other polymer; in the “intermediate” zone for labels attached near but not at the surface of, for example, a lipid bilayer; and in the “bulk” zone for small molecule nitroxides freely dispersed in water or certain highly charged polymers such as DNA.
with the protein surface but slows the overall protein tumbling
time by increasing the bulk water viscosity by about 10-fold at
21% (w/v) concentration. Interestingly, the values for $k_{low}/$
$k_{low,bulk} \times 1/e_{b}^{\sigma_{bulk}}$ and $e_{c}/e_{b,\text{bulk}}$ are all, within error, unaltered,
suggesting that there is no bound water population whose effect
is masked due to fast protein tumbling in the absence of Ficoll
70. The contribution from fast moving water, as reflected in $k_{c}/$
$k_{low,bulk}$ also remains unaltered and, in keeping with previous
observations on lipid surfaces, remains unaffected by the
increase in the bulk solvent viscosity induced by Ficoll 70, thus
confirming that this polymeric viscoelastic does not interact with
the GroES surface.

Interestingly, the value of $k_{low}/k_{low,bulk}$ for the GroES/SR1
complex is found to be somewhat higher (and may exceed the
error of measurement) than the corresponding values for GroES with or without Ficoll 70 (Figure 5). The increase in $k_{low}$
leads to a slightly larger apparent retardation factor, thereby
indicating slower hydration dynamics (see $e_{c}/e_{b,\text{bulk}}$ in Figure 5).
To understand the subtle meaning of these changes, we recall
that ODNP is sensitive to fluctuations in the spin–spin dipolar
interaction between water and the spin label that is attached to
the surface of GroES. The value of $k_{c}$ samples fluctuations with
time constants of tens of picoseconds and faster (i.e., 10 GHz
fluctuations). Fluctuations on this time scale are typically
associated with water molecules freely diffusing past a spin
label. Therefore, the change in $k_{low}$ observed here does not
reflect a change in the dynamics of freely translationally
diffusing hydration water since such a change would also alter
the value of $k_{c}$. Rather, a selective increase in $k_{low}$ as observed
here, indicates an increase in slower fluctuations, with time
constants as low as 10 ns (i.e., 15 MHz fluctuations).
Fluctuations on this time scale can arise either when, for
example, water molecules near the spin label bind partially (for
ns or tens of ns) to the surface of GroES as it tumbles in solution or when water molecules chemically exchange with
labile protons on the protein surface near the spin label. Thus,
it is possible that GroES/SR1 either might trap a limited
number of partially bound water molecules or may engage the
water in chemical exchange. Because the value of $k_{low}$ is the
same (within error) for GroES with or without Ficoll 70, this
limited population of bound or exchanging waters would only
be present in the chaperone complex and not on the surface of
free GroES. However, most importantly, because the change in
$k_{low}$ is small (2-fold at most), we can assume that these changes
indicate the presence of relatively few bound or exchanging
water molecules. Even these small changes do not arise from
changes in the freely translating water inside the nanocavity, as
indicated by the consistent $k_{c}$ value.

We conclude that the SL-GroES surface is very weakly
hydrated with highly mobile surface water, with no contribution
of surface bound water, thus representing an unusual protein
surface. There are indications that, upon formation of the SL-
GroES–SR1 complex, a very select and small number of water
molecules either bind partially to the cavity surface or engage in
chemical exchange with it. However, it is clear that the majority
of the water molecules continue to exhibit the same unusually
high mobility and weak hydration even when confined inside
the SL-GroES–SR1 cavity. This implies that the repulsive
hydration barrier for a substrate to approach the GroES surface
is very small and that the substrate experiences a bulk water-like
environment, even upon confinement within the cavity of the
SL-GroES–SR1 complex.

A previous computational study\textsuperscript{12} suggested that the folding
potential of proteins within the chaperonin cavity is enhanced
owing to the hydrophilicity of the cavity inner surface, as
measured by the density of surface water. When employing
ODNP methods, a high hydrophilicity of a protein surface
would be reflected in retarded surface water diffusivity because of
the attraction of water to the protein surface. However, we
observe rather unusually fast dynamics of water on the cavity-
fac ing surface of GroES, both when it is free and when it is in
complex with GroEL. ODNP-NMR does yield very slightly
different results for the GroES/SR1 complex due to the
presence of a small number of bound water molecules or labile
protons on the inner surface of the cavity but does not yield
results suggesting an overall slowing of the hydration water.
The fast dynamics seen here have been seen for the surfaces of
unstructured polymers\textsuperscript{5,46} but have not been observed before in
cases of proteins and lipid membranes (see Figure 6). These
unexpectedly fast diffusion dynamics of the surface hydration
water implies a low repulsive barrier for the substrate to
approach (and leave) the GroES surface as well as a low folding
potential for the substrate near the GroES surface. This
suggestion that the GroES lid confers a low protein folding
potential is in agreement with the finding\textsuperscript{47} that replacing
Tyr71 in GroES with charged residues enhances the GroEL-
assisted folding of GFP. Our observation that the cavity-facing
surface of the GroES lid has a low folding potential is also in
agreement with the report that nonfolded substrate proteins
can approach the lid and escape from the cage.\textsuperscript{48}

\section*{CONCLUSIONS}
In this study, the properties of the chaperonin cavity-confined
water were studied using ESEEM and ODNP by attaching a
spin label to a cysteine in the Tyr71 → Cys GroES mutant.
This residue is fully exposed to bulk water in free GroES and
can probe the confined water in the upper region of the cavity
in the GroEL–GroES complex. Previous work has shown that
replacement of Tyr71 in GroES with positively or negatively
charged residues enhances GroEL-assisted GFP folding,\textsuperscript{47}
thereby indicating that the position we labeled senses a region
of the cavity that is of functional importance. Our main findings
are that both the density and the dynamics of the water in the
vicinity of the spin label are the same in free and SR1-bound
GroES, and that the properties of the cavity-confined water are
similar to those of bulk water. These findings are consistent
with the claim that the folding process inside the GroEL cage is
similar to that in bulk solution, i.e., that the GroEL cavity is a
“passive” cage in which folding is not accelerated\textsuperscript{49}
and may even be slowed down.\textsuperscript{50} It should be borne in mind, however,
that the dynamics of the surface water closer to the bottom of
the GroEL cavity may be vastly different (e.g., slower) than
those of water at the top. Future studies need to be designed
for probing the properties of water at the bottom of the cage
and in the presence of nonfolded substrates.

\section*{ASSOCIATED CONTENT}
\section*{Supporting Information}
Figure showing correlation between the spin-label-induced
relaxation rate and the ODNP cross-relaxation rate. This
material is available free of charge via the Internet at http://
pubs.acs.org.

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