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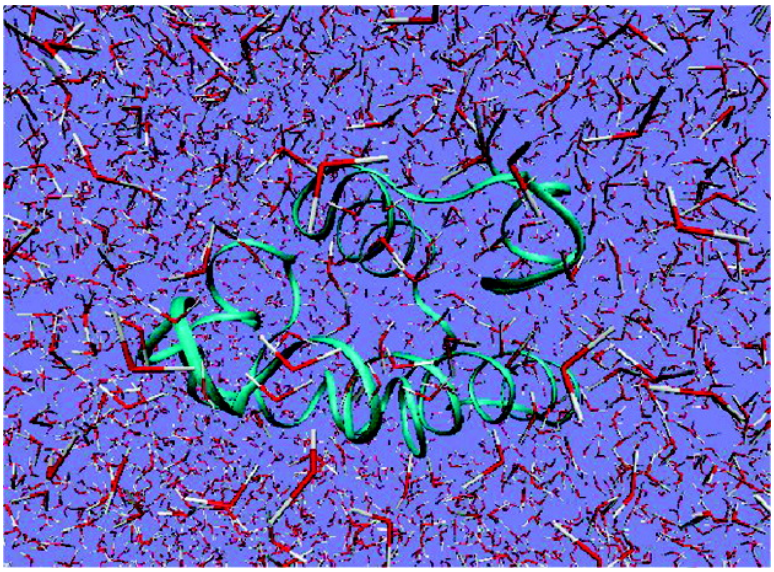

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Protein Sequence- and pH-Dependent Hydration Probed by Terahertz Spectroscopy

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Solvation free energy changes induced by protein folding and function are comparable to the corresponding overall free energy changes. Yet the structure and dynamics of the protein itself have received more attention than the solvent because they are easier to probe. Here we use terahertz (far-infrared) spectroscopy to probe directly the effect of mutations and solvent pH on the solvent shell-protein interaction. We study absorption spectra of the 80 residue five helix bundle λ^*_{6-85} in the 2.1−2.8 THz region. We find that the pseudo-wild-type has a much more pronounced effect on long-distance solvation water than mutants replacing a single polar glutamine side chain with aromatic residues. This is true both in the context of enhanced and decreased helix stability. Unfolding the pseudo-wild-type at pH 2 likewise reduces the long distance solvation effect. Thus terahertz spectroscopy, in combination with site-specific mutations, can detect changes in both local and global solvation dynamics around proteins.

Several recent studies have addressed the molecular-level dynamics of hydration layers from the protein surface to the bulk.[1] Using X-ray and neutron diffraction, Head-Gordon and co-workers found for low concentration of the NALMA peptide an additional elastic component is activated, which is attributed to a coupling between inner and outer hydration layers.[2] Molecular dynamics simulations for villin headpiece in aqueous solution yielded a change in the density of water near the protein upon unfolding and a correlation of the water dynamics with the folding process.[3]

THz spectroscopy is a new tool to study the solvation dynamics by probing the coupled collective modes of solute and solvent.[4] It is experimentally challenging because of the strong THz absorption of water. Using a free electron laser, Plaxco, Allen and co-workers showed that terahertz absorption decreases linearly when large concentrations of protein are added to the solution.[5] Such behavior indicates that the solute molecules replacing the water have a lower absorption within this frequency range. A coupling of THz hydration dynamics and protein dynamics was also suggested by spectroscopy of hydrated bacteriorhodopisin films.[6]

We have devised table-top THz sources capable of penetrating the bulk of aqueous solutions.[7] Our previous study showed that the absorption is nonlinear at low concentrations (1−5 mM range), indicating that solvation water molecules exhibit enhanced absorption in a wide (>10 Å) solvation shell.[8] Molecular dynamics simulations of the dipole correlation function of the solvation water supported the hypothesis that the THz absorption of the hydration shell could depend on the distance between the proteins in agreement with studies by Pettitt and co-workers that show retarded dynamics for water between nearby solutes.[9]

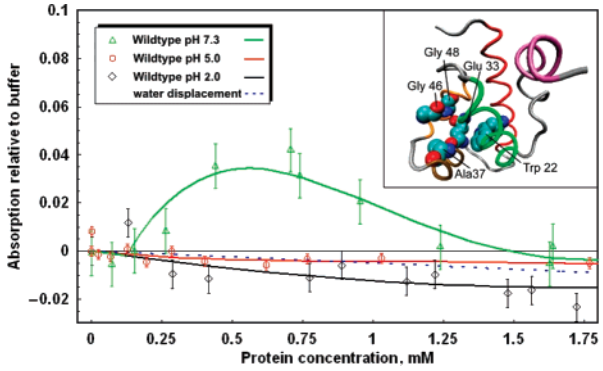

Figure 1. Difference in the integrated THz absorption coefficient (2.1−2.8 THz) of λ^*_{6-85} at pH 2.0, pH 5.0, and pH 7.3 relative to $\alpha_{rel} = [\alpha(\text{protein})-\alpha(\text{bulk})]/\alpha(\text{bulk})$ plotted against concentration. Shown is the average of several subsequent measurements at the same concentration along with the statistical error. The main error source is the refilling of the sample cell. Further details of the experimental setup can be found in ref 4. The temperature is kept at 20 °C. The inset shows the structure and the mutation sites. The absorbance for the native protein (pH 7.3) depends nonlinearly on concentration in this region, indicating overlapping hydration shells. In contrast the concentration dependence of the THz absorption of the destabilized protein (at pH 2 and 5) resembles the predicted decrease due to the replacement of water molecules by the proteins.

Here we compare the concentration-dependent THz absorption of the λ^*_{6-85} pseudo-wild-type (Figure 1 inset) with engineered surface mutants and with low pH data. As in previous work, we found that the absorption coefficient of the aqueous buffer increases approximately linearly over the 2.1−2.8 THz frequency range, from $\alpha \approx 350$ to 380 cm^{-1} (Figure 2 inset, for the buffer used and insensitivity of spectra to it, see ref 8). Whether the protein + solvent absorption is higher or lower than the solvent absorption alone depended on the protein concentration. SAXS measurements ruled out excessive aggregation of the native protein.[10] This led to the question of whether the absorption spectrum could act as a sensitive marker for local as well as global perturbations of the solvent structure around a protein.

As a global perturbation of protein solvation, we lowered the pH from 7.3 to 5 to 2. The absorption of the buffer alone is constant over this pH range. The protein has gone partway through the unfolding transition at pH 2 (as monitored by cicular dichroism and fluoresence wavelength shift; Supporting Information Figure S1). ANS binding to the proteins is enhanced at lower pH (2 and 5), indicating a more exposed hydrophobic surface area.

We observe a strong pH dependence of the absorption. At pH 7.3, addition of protein to the buffer increases the absorption coefficient 0.5−1 mM concentration (Figure 1), whereas at pH 2

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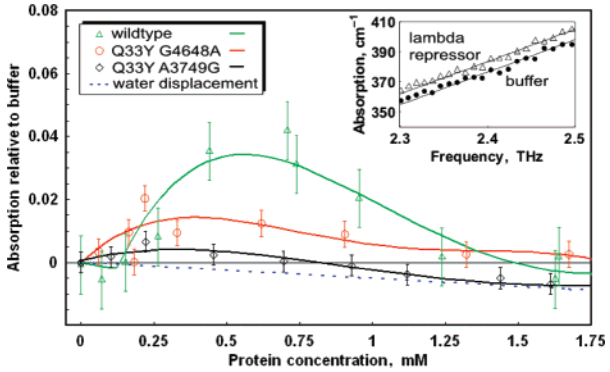

Figure 2. Comparison of the integrated THz absorbance (between 2.1 and 2.8 THz) of the pseudo-wild-type lambda repressor with three mutants of the protein at pH 7.3. The inset shows the frequency dependence of the THz absorption for buffer and the solvated protein at a given concentration of 0.37 mM, 20 °C. The nonlinear concentration response is most pronounced for the wild type. It is less significant for the helix-stabilized mutants. The mutant Q33Y/A37,49G deviates the least from a simple solvent displacement model (dotted line).

and 5, the protein solution has almost the same or slightly lower absorption coefficient than aqueous buffer.

The non-monotonic behavior observed at pH 7.3 cannot be explained by an effective two-component model: λ^*_{6-85} displaces about 7400 Å^3 of buffer.[8] If the protein itself has a decreased absorption at 2.4 THz compared to water as was found previously,[5] we would expect a linear decrease of the THz absorption coefficient with concentration, as discussed by Xu et al.[5] and in our earlier work.[7] In the case of a completely transparent protein which displaces water, we expect a decrease according to the dotted line in Figure 1. The pH 7.3 data indicate that the absorption coefficient of hydration water is enhanced by the presence of protein. This enhancement at low concentrations indicates a dynamical solvation shell of >10 Å thickness around the protein.[8] At pH 2, the absorption lies slightly below the dotted line that posits a completely transparent protein, indicating hydration water with an unusually low absorption coefficient. The pH 5 data follow the dotted line accurately, but this appears to be due to a compensation between solvation water of enhanced absorptivity (native protein) and solvation water of reduced absorptivity (pH 2 unfolded state).

To study a site-specific solvation effect on the THz spectrum, we substituted Gln33 → Tyr by site-directed mutagenesis, replacing the highly polar glutamine side chain ($CH_2CH_2CONH_2$) by a less polar aromatic side chain. When coupled with Ala-Gly mutations (A37,49G) that greatly destabilize the protein,[11] the Tyr mutant shows a concentration dependence similar to the low pH proteins, with only a remnant of a concentration maximum. When coupled with a helix-stabilizing mutation (G46,48A), about half the maximum in absorption relative to buffer is restored when compared to pH 7.3. Thus a quadruple mutation (A37,49G to G46,48A) that stabilizes helices in λ^*_{6-85} is not sufficient to completely offset the effect induced by a single point mutation at position 33. The results are summarized in Figure 2. Terahertz absorption can thus be used in conjunction with site-directed mutagenesis to probe local interaction of protein surfaces with their solvent shells.

To complement the experimental data, we studied the approximate dynamics of the protein and explicit solvent water by molecular dynamics (MD) simulation. To obtain a clear trend we compared native and denatured proteins, the two extremes in flexibility and hydrophobic surface exposure probed in Figure 1. Figure 3 shows the results for the predicted average lifetimes of H-bonds for λ^*_{6-85}. Water molecules around the denatured state show retardation of the dynamics, caused by the exposure of

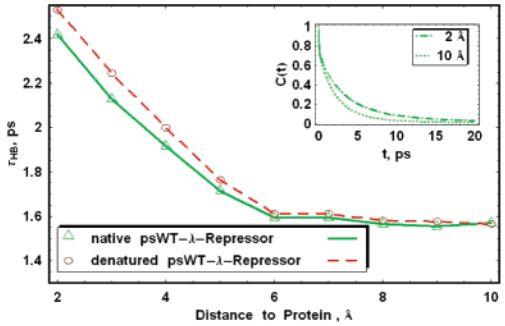

Figure 3. Comparison of the average lifetimes of water−water H-bonds in the vicinity of native and denatured lambda-repressor calculated from MD simulations. The inset shows the H-bond lifetime correlation functions of the native protein at 2 and 10 Å distance, where the average H-bond lifetime converges to the bulk value.

hydrophobic residues of the denatured protein to the water. The inset shows that the retardation is distance-dependent making differences in concentration dependence plausible for the native and the denatured protein. Over longer time scales, differences in flexibility in the MD simulations[12] were also observed for mutants. Calculation of the THz spectrum by normal-mode analysis revealed differences for the native and partially denatured protein, but did not yield consistent trends in the THz absorption with point mutations or lower pH values; thus the experimental results still remain to be explained.

In summary, we have shown that global perturbations of the protein hydration shell by pH and local perturbation by surface site-specific mutation both produce significant changes in the terahertz absorption spectrum of aqueous protein. Such changes can be used in the future as sensitive probes of protein−solvent dynamics, opening up the possibility of using THz absorption as a probe for protein folding kinetics and functional dynamics measurements. The development of quantitative models for the THz spectra will make it possible to understand local solvation of proteins at the molecular level.

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Supporting Information Available: CD spectra of the least stable mutant, λ^*_{6-85} Q33Y/A37,49G at pH 7.3 and at pH 5. In addition we show the CD of unfolded λ^*_{6-85} at pH 2. This material is available free of charge via the Internet at http://pubs.acs.org.

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