Terahertz Spectroscopy

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Real-Time Detection of Protein–Water Dynamics upon Protein Folding by Terahertz Absorption Spectroscopy**

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Recently, there has been a growing interest in probing not just the dynamics of self-assembling macromolecules but the dynamics of their solvation shells as well. Dielectric, Raman, fluorescence, and NMR spectroscopies, neutron scattering, and crystallography all provide insights, but only terahertz absorption spectroscopy (wavelength range 0.1–1 mm; 1 THz = 1 ps^{-1}) directly probes the picosecond solvent dynamics over any desired time scale and is sensitive to hydration layers far from the molecular surface.[1]

Protein folding is a self-assembly process in which solvent motions play a critical role. The free-energy contributions of the protein and of the solvation water are comparable during folding,[2] and water dynamics are perturbed by the protein beyond two solvation layers.[3,4] Yet folding has been probed in the past mainly with an emphasis on the backbone and sidechains of the protein itself. Can we directly probe solvent reorganization during secondary structure or hydrophobic core formation? Terahertz sources have become powerful enough to study directly the absorption spectroscopy of biomolecules in aqueous buffer.[5–10]

We recently showed that THz absorption is sensitive specifically to solvation water around proteins.[11,12] At the same time, time-domain THz spectroscopy has been applied in absorption and emission to study picosecond dynamics on the time scale of the THz pulse itself,[13,14] and THz absorption has been used to monitor slow kinetics.[15]

Herein we introduce kinetic THz absorption (KITA) for investigation of solvation dynamics during folding. KITA monitors the changing THz electric field pulse shape on the picosecond time scale Δt, as a chemical reaction proceeds on a longer time scale t.

We apply KITA to measure the changing protein–hydration-water dynamics during the fast refolding of ubiquitin. Ubiquitin is a 76-residue, predominantly β-sheet protein that has long been used as a prototype for folding kinetics studies.[16] The folding kinetics detected by KITA are compared to small-angle X-ray scattering (SAXS), tryptophan fluorescence, and circular dichroism (CD), revealing that in the 0.1–1 THz range, the solvation dynamics are coupled to secondary structure formation (including a switch from solvent–protein towards more protein–protein hydrogen bonds) and to protein compactification, whereas formation of native-like tertiary structure around the tryptophan takes place on a hundred-fold slower time scale.

Our apparatus is illustrated in Figure 1. It resolves the THz pulse with sub-picosecond time resolution and measures with millisecond time resolution the changes in the pulse caused by changing absorption of hydration shells and their associated proteins during refolding initiated by a mixer. Pulses spanning the 0.1–1 THz frequency range were used. By scanning the time delay of the THz pulse relative to the gating pulse, the THz electric field is mapped out precisely. By changing the "kinetic" time between stopped-flow and THz pulse, the kinetics of folding are mapped out. (See the

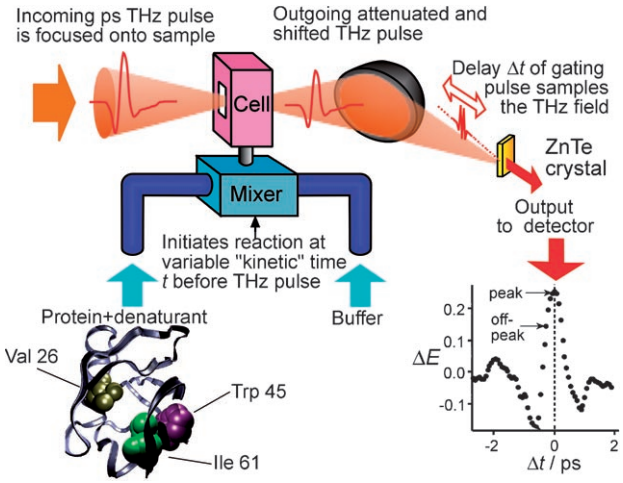

Figure 1. KITA setup: THz pulses pass through a stopped-flow cell, where a mixer combines denatured ubiquitin with denaturant-free buffer to start refolding. The shape of the transmitted THz electric field is detected using a ZnTe crystal and an 800 nm gating pulse delayed by Δt. The difference ΔE of the electric field between denaturant-free 1.5 mM protein solution and buffer is shown. For kinetics, the THz pulse is detected near the maximum electric field, and the mixer is scanned in time t with respect to the THz pulse.

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Supporting Information for further details of the KITA apparatus.)

For the fluorescent Phe45Trp mutant of ubiquitin dissolved in buffer (Ub*; 0.5–1.5 mM), we previously observed a stronger absorption of 2.5 THz light than for buffer or for the protein alone. We showed that altered water dynamics up to 18 Å from the protein surface account for the excess absorption.[4,11,12] We suggested that the excess absorption of hydration water at 2–3 THz occurs because the protein–water coupling induces a shift of absorbance from sub-THz to higher-frequency modes.[11]

We now find that ubiquitin solution absorbs less than buffer in the 0.1–1 THz region, in agreement with this suggestion. Fourier transform of the THz electric fields from the time to the frequency domain yields the transmitted intensity for protein solution and pure buffer (Figure 2). The

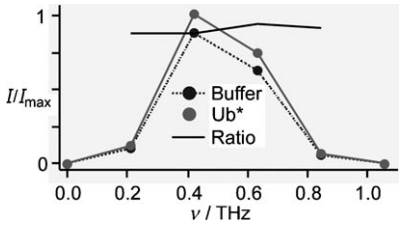

Figure 2. 1.5 mM Ub* solution transmits more THz intensity *I* than pure water/ethylene glycol buffer. The ratio is nearly constant.

protein and its hydration water typically absorb 10–20% less than the bulk water they replace. The net difference between buffer and protein is nearly constant from 0.2 to 0.8 THz, so the KITA-detected kinetics are not expected to be wavelength-sensitive in this range.

Next, we used the change in transmitted THz electric field between folded and unfolded protein solutions (typically 3–5%) to monitor folding kinetics. We diluted a solution of unfolded protein in 6 M guanidinium buffer to 0.86 M guanidinium, thus inducing folding. Refolding kinetics monitored by KITA provide a direct window on protein–solvent rearrangements during folding, such as the breaking of backbone–water hydrogen bonds and their replacement by backbone–backbone hydrogen bonds. Figure 3 (left) shows schematically how KITA probes the evolution of collective protein–hydration water dynamics during refolding kinetics. THz pulses probe the sample with delays between 0.05 and 5 seconds after the protein has been mixed into low-guanidinium buffer, tracing out refolding kinetics. We collect a buffer sample for reference and plot the kinetics either as the ratio of protein transmission to buffer transmission (Figure 3, right) or as the difference between the two (Figure 4). Because the change in absorption between protein solution and buffer is relatively small, both methods yield fits with the same time constant. The detectable kinetics are in the millisecond range under all conditions we measured.

The nearly wavelength-independent absorption in Figure 2 implies that the kinetics we detect should not depend on which part of the THz pulse we probe, as indeed

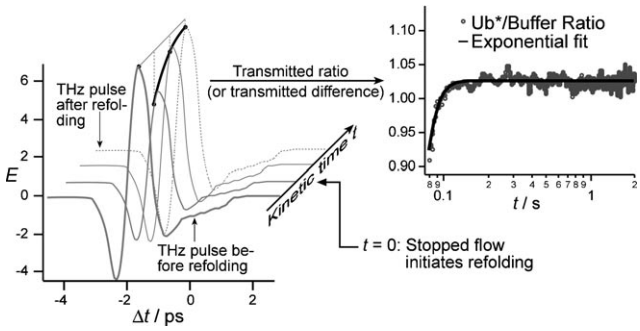

Figure 3. Left: electric field *E* of THz pulses. As the mixer is scanned in time *t* with respect to the THz pulse, the field changes because the folded protein solution has different THz absorbance and refractive index than the unfolded protein solution. Right: the ratio of protein to buffer signal reflects the refolding kinetics of Ub* (−20 °C, water/ethylene glycol buffer).

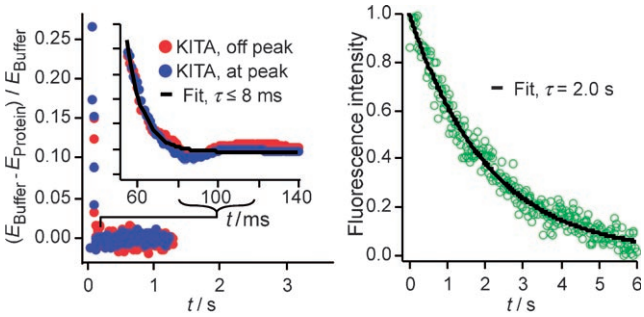

Figure 4. Ub*V26A kinetics. Left: Terahertz transmission on and off the transmitted electric field peak yields identical millisecond kinetics at −20 °C. Right: Fluorescence-detected kinetics are much slower.

we find. Figure 4 shows the folding kinetics for a Val26Ala mutant of Ub*, detected at the peak and off the peak of the THz pulse, as indicated by the arrows in Figure 1. Within our measurement uncertainty, the refolding kinetics are identical. Similar results were obtained for the other proteins we studied.

The folding kinetics of Ub* and of its mutants turn out to be highly probe-dependent. It is clear from the time scales in Figure 4 that THz transmission approaches the native equilibrium value of Ub*Val26Ala nearly two orders of magnitude faster than tryptophan fluorescence. Within the signal-to-noise ratio achieved for Ub* and its mutants, we were unable to observe any slow phase by KITA (e.g. Figure 3). Thus KITA below 1 THz reports on protein–solvent dynamics that equilibrate well before the tryptophan is packed into a native-like environment.

We also compared results from KITA to CD and SAXS data on the refolding kinetics of Ub* and Ub*Ile61Ala.[17,18] To compare directly with the prior CD and SAXS experiments, we measured KITA under the same solvent conditions (40 mM phosphate buffer, 45% ethylene glycol in water buffer at pH 5.9). Figure 5 compares KITA, fluorescence, CD, and SAXS data for Ub*. (Ub*Ile61Ala is similar and is shown in the Supporting Information.) KITA has only a fast millisecond phase. Fluorescence has only a slow phase. CD and SAXS show both phases. The altered dynamics of

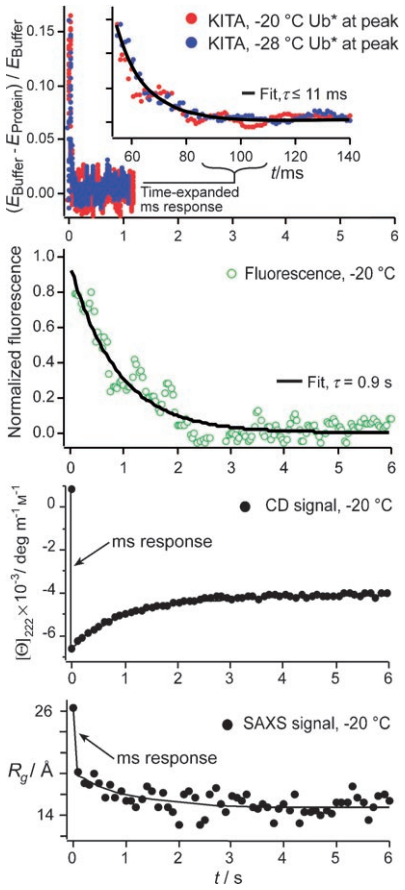

Figure 5. KITA, fluorescence, CD, and SAXS refolding kinetics of Ub*. The bottom two panels are adapted from reference [17]. Because of the dead time, the KITA fit is an upper limit.

hydration water and protein detected at 0.5 THz go hand in hand with a rapid overshoot of the CD signal at 222 nm. The CD overshoot is due to formation of excess helical structure relative to the native state of ubiquitin, with the accompanying reduction in the number of hydrogen bonds from the protein backbone to the hydration shell.[4,18] SAXS measurements show that Ub* and Ub*I61A undergo a rapid collapse on the millisecond time scale, but SAXS data of Ub* also show a slow signature that matches the fluorescence data. Thus the rearrangements of the solvent network detected by KITA occur during initial collapse and formation of early secondary structure, whereas further rearrangements required by native packing are not picked up by KITA at 0.1–1 THz.

Comparison of the KITA data for Ub*, Ub*V26A, and Ub*I61A shows that native protein flexibility has no systematic effect on early THz kinetics. Ub*V26A and Ub*I61A display single sidechain truncations of nonpolar residues. The valine residue is completely buried, while isoleucine 61 is largely buried. Ub*I61A fits to a slightly slower exponential decay than Ub*; Ub*V26A fits slightly faster. All are in the range 18 ± 10 ms. Considering the 50 ms dead time of the stopped flow, these differences are not significant. Thus the early kinetics detected by KITA are not strongly affected by mutations that significantly destabilize the native state.

Fast Ub* folding dynamics have no strong temperature dependence detected by KITA. Figure 5 (top) compares the early THz folding kinetics of Ub* at two different temperatures, −20 and −28 °C (chosen to allow direct comparison with existing SAXS data[18]). The traces fit to the same millisecond exponential decays within fitting uncertainty; the same is true for other mutants of Ub* (data not shown). We can use this finding to set limits on the activation energy for rearranging the solvation water network during early folding events and find that the water network rearrangements and large-amplitude protein motions probed by KITA have a small activation energy, less than 15 kJ mol^{-1}, whereas the later stage of folding monitored by fluorescence has a barrier of about 27.5 kJ mol^{-1}. (Details of the calculation are given in the Supporting Information.)

Conceptually, the interpretation of the THz kinetics experiment is straightforward. For the 2–3 THz data, we proposed a coupling of protein surface flexibility and hydration shell to explain the sensitivity of absorbance to sidechain truncations in the core of the protein.[12] In contrast, KITA kinetic measurements at 0.1–1 THz are not sensitive to changes in protein flexibility that result from sidechain truncations, because the protein is just beginning to fold and does not yet have a well-defined surface. This finding indicates that a different mechanism influences the THz spectrum at lower frequencies early during the folding process.

A comparison with circular dichroism data allows us to propose a tentative mechanism. The time scale observed by KITA is in line with the 6 ms upper limit set by circular dichroism spectroscopy on a fast phase that forms excess helical structure. Considering that during this time span, hydrogen bonds from the protein backbone to water are broken and then remade as intramolecular hydrogen bonds to form secondary structure, the agreement between circular dichroism and KITA is entirely plausible. We thus assign the KITA relaxation kinetics to the formation of intermolecular hydrogen bonds early during protein folding.

If this interpretation is correct, future investigation of KITA in deuterated water would be interesting. We predict that the THz defect we observe at 0.5 THz and the excess we previously observed at 2.5 THz would red-shift and would be very sensitive to the use of deuterated solvent. Likewise, monitoring the kinetics in water in the higher-frequency 2–3 THz band would be interesting. Since this wavelength region is more sensitive to surface flexibility differences induced in the native state by core mutations,[19] we might be able to detect later stages (between 50 ms and 2 s) in which a native-like protein surface forms, especially at high protein concentrations. Finally, we suggest the need for explicit solvent molecular dynamics simulations that compare the solvation of denatured and folded states of ubiquitin by computing the absorption at 0.5 THz and 2.5 THz from the dipole–dipole autocorrelation function.[1]

The agreement between the CD (sensitive to secondary structure) and KITA (sensitive to the protein–hydration-water interaction) time scales shows how closely protein dynamics and solvent dynamics interact during folding. Although our measurements do not make a cause-and-

effect distinction between protein and solvent dynamics, our results are in agreement with the hypothesis proposed by Frauenfelder and co-workers that some protein dynamics are slaved to solvent motions. The motions we observe by KITA would be the so-called alpha-fluctuations proposed by Frauenfelder.[20]

Experimental Section

The plasmids for ubiquitin mutants were made as described in reference [19] from the original Ub* plasmid (provided by Tracy Handel),[21] which has a Phe45Trp mutation to introduce a fluorescent marker. Plasmids for each mutant were inserted into the pET-15b vector and expressed in Rosetta TM (DE3) pLysS cells (Novagen Inc). Cells were lysed with a French press. Collected supernatants were bound to a CM-52 cation exchange column and eluted with a linear salt gradient from 0 to 1M NaCl for purification. Additional purification was performed with Amicon 3 kDa and 30 kDa membranes (Fisher Scientific). The purity of ubiquitin mutants was checked by electrospray ionization mass spectroscopy and SDS-PAGE gel electrophoresis. Protein concentrations were determined by UV absorption spectroscopy (Shimadsu UV-1650 PC) at 280 nm. Protein was dissolved in a ethylene glycol/water buffer (45:55 by volume) with 40 mM sodium phosphate at pH 5.9. Guanidinium denaturation curves are shown in reference [19].

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