A Bioassay Based on the Ultrafast Response of a Reporter Molecule

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The capability of using ultrafast detection technologies for a fast analysis of biomolecular reactions has been explored. As an example, the ultrafast response of tetramethylrhodamine (TMR)–labeled bovine serum albumin (BSA) as a function of different extents in proteolytic cleavage was investigated. The authors compared 4 samples of masses differing over several orders of magnitude: untreated, TMR-labeled BSA (66 kDa), TMR-labeled BSA treated with elastase (6-33 kDa) and with subtilisin (< 3 kDa), and the pure label TMR (0.4 kDa). A direct comparison with gel electrophoresis revealed that various ultrafast parameters give robust information about the progress of the proteolytic cleavage. The authors found the ratio of the transient absorption signal observed at 0 psec and 50 psec after excitation ($\lambda_{\text{Pump}} = 540$ nm, $\lambda_{\text{Probe}} = 570$ nm) to be the most precise parameter for determining the cleavage. This parameter allowed determining the mass accurately within 1 sec (Z’ factor of 0.83) or 600 msec (Z’ factor of 0.64), measuring time per sample. This indicates that many of the known ultrafast detection technologies might be used for monitoring biochemical reactions, probably even without any labeling procedure. The authors also discuss briefly which ultrafast processes contribute to the signals and how they are affected by changes in the biomolecular environment. (Journal of Biomolecular Screening 2007:341-350)

Key words: bioassay development, ultrafast spectroscopy, proteolytic cleavage, high-throughput screening, fluorescence markers

INTRODUCTION

Fluorescence lifetime assays are very well established for the determination and analysis of various biochemical reactions. The beauty of the fluorescence lifetime analysis is that it depends only on the changing fluorophore kinetics in a changing biomolecular environment and not on the actually detected fluorescence intensity. It is thus independent from artifacts typically associated with fluorescence intensity techniques such as variations in the concentration due to pipetting errors or scattering in cell imaging. For this reason, fluorescence lifetime readouts are extremely accurate and give excellent assay parameters ($Z’$ factors), which are even suitable for ultra-high-throughput screening (ultra-HTS). Typically, a measurement time of ~100 msec per sample is sufficient. The major drawback of fluorescence lifetime assays is that possible effects of a certain biochemical reaction onto the fluorescence lifetime cannot be predicted. Therefore, various different fluorescent labels and labeling sites have to be tested, normally, to develop a suitable assay. Very often, no sensitivity of a fluorescent lifetime assay can be found at all.

Fluorescence lifetime assays usually reflect excited state population dynamics of the fluorophores occurring on the order of nanoseconds. Nevertheless, after excitation, fluorophores first undergo very complex intra- and intermolecular relaxation processes on the femto- to picosecond timescale before light is emitted via fluorescence. These ultrafast processes are usually not resolved by a standard fluorescence lifetime analysis. However, it is well known that this very complex ultrafast femto- and picosecond response of molecules depends very sensitively on details in the molecules’ environment. It can be expected that the ultrafast response senses biochemical reactions even in cases where a fluorescence lifetime assay is not sensitive. Processes such as intramolecular vibrational redistribution (IVR), vibrational energy transfer (VET) to the solvent, dielectric response, and dipole reorientation can be dramatically varied in amplitude and timescale by enzymatic modifications, receptor-ligand binding, or other biochemical reactions. The measurement of the ultrafast kinetics of molecules on a femto- to picosecond timescale has in principle the same advantages as fluorescence lifetime measure-
ments because the observed kinetics are similarly independent from artifacts such as variations in the concentration. A typical technique to measure ultrafast dynamics is pump probe spectroscopy. A first ultrafast pump pulse (typically shorter than ~100 fsec FWHM) excites the fluorophore. After a certain time delay, a second ultrafast probe pulse of the same or another wavelength measures changes in the transient absorption of the molecule. Even though the necessary ultrafast laser systems have reached a level of reliability that allows using them in a full-automated robotic environment, to our knowledge, ultrafast technology has not been used for a fast determination of biochemical reactions. The motivation of the present report is to elucidate to which extent the complex ultrafast behavior of fluorescence markers or the biomolecules themselves can be used to monitor biochemical reactions as fast and sensitively and probably even more specifically than by fluorescence lifetime techniques.

In this context, we present a systematic investigation of the influence of enzymatic cleavage reactions on the ultrafast response of bovine serum albumin (BSA) labeled with the fluorescence dye tetramethylrhodamine (TMR). Different proteases were used, which resulted in different sizes of cleaved fragments. A comparison of gel electrophoresis with the measured ultrafast dynamics revealed various robust parameters that correlated well with the extent in proteolytic cleavage. With measurements at only a few points of the ultrafast kinetics, it is possible to measure the cleavage at acquisition times as necessary for HTS while still preserving high accuracies (Z factor > 0.5). The model system presented demonstrates the potential of using the complex ultrafast response of reporter molecules for a fast and sensitive elucidation of biochemical reactions. Because classical ultrafast pump probe measurements do not necessarily require a fluorescent label, even label-free measurements of biomolecular reactions might become feasible. To gain an insight into the expected changes of ultrafast signals, we discuss briefly which ultrafast processes contribute to the signals and how they might be affected by changes in the biomolecular environment.

MATERIALS AND METHODS

Samples and enzymatic cleavage reaction

The fluorophore tetramethylrhodamin 5- and 6-isothiocyanate (TMR) and the TMR-labeled protein BSA were purchased from Invitrogen (product codes T 490 and A 230; Carlsbad, CA) and used without any further purification. BSA had a labeling degree of about 5 TMR molecules per protein. The enzymes subtilisin A from Bacillus sp. and elastase, from porcine pancreas, were purchased from Sigma–Aldrich (product codes P 5380 and E 7885; St. Louis, MO). All measurements and reactions were done in phosphate-buffered saline (PBS) buffer solution. For the pump probe experiments, all enzymatic cleavage reactions were done at room temperature 20 min prior to the measurements. A substrate concentration corresponding to ~5 µM BSA (prior to digestion) was used. BSA and its cleaving fragments were characterised by gel electrophoresis under discontinuous denatured conditions (120 V, 2 h) using Coomassie staining. Three fragments of about 23, 27, and 33 kDa dominated the cleavage products using elastase. Some smaller bands indicated cleaving products of about 6 to 7 kDa. Using subtilisin resulted in fragments smaller than 3 kDa. The molecular mass of BSA and the free label were 66 and 0.4 kDa, respectively. In addition, fluorescence and absorption spectra were recorded on a Varian Cary Eclipse (Varian, Inc., Palo Alto, CA) fluorescence spectrophotometer and PerkinElmer Lambda 9 UV/Vis/NIR spectrometer (PerkinElmer, Wellesley, MA), respectively.

The ultrafast pump probe laser system has been described in detail previously. Briefly, the laser system consisted of an ultrafast laser oscillator (Coherent Verdi Duo, Coherent, Inc., Santa Clara, CA) seeding a Coherent RegA 9000 amplifier.

For the present investigation, the amplifier pumped an Optical Parametric Amplifier (Coherent OPA 9450) at a repetition frequency of 125 kHz. The OPA has a so-called signal beam output tunable from 500 to 700 nm and a white light beam output covering wavelengths from ~460 to 1200 nm. The signal beam pulses had pulse widths of approximately 100 fsec and pulse energies of several tens of nJ. The signal beam was used as pump beam and tuned to wavelengths, \( \lambda_{\text{pump}} \), of either 540 or 560 nm. This pump beam was focused into a flow cell containing the samples (2-mm optical path length, Fig. 1). The
Bioassay Based on Ultrafast Response of a Reporter Molecule

OPA white light output was used as probe beam and was focused into the same spot in the flow cell as the pump pulse. The probe pulses passed a motorized translation stage (ILS 200HA, Newport) to adjust the time delay between pump and probe pulses. The desired probe wavelength range was pre-selected by a variable spectral filter (Veril INT, 400–1000 nm). After passing the flow cell, the probe pulse was directed into a monochromator (model 500ISSM, Chromex, Albuquerque, NM) to select a defined detection wavelength, \( \lambda_{\text{probe}} \). Changes in the probe light intensity due to transient absorptions of the sample were monitored by a fast photodiode. A log-in amplifier (EG&G Brookdale Electronics, Princeton Applied Research, Princeton, NJ) was used to amplify the modulation in the transient absorption signal in the probe beam, which was caused by an optical chopper placed in the pump beam path. Changing the time delay between pump and probe beam from –100 to +600 psec in steps of 200 fsec, 1 psec, or 5 psec allowed us to determine ultrafast decay curves as plotted in Figure 2.

RESULTS

For the ultrafast pump probe measurements, samples of the free dye TMR, a sample of TMR-BSA cleaved by subtilisin, a sample of TMR-BSA cleaved by elastase, and a sample of the noncleaved TMR-BSA were prepared. Subtilisin is cleaving almost all peptide bonds, whereas elastase only cleaves at alanine. Thus, the 4 samples corresponded to a series of increasing molecular masses of the residues attached to the fluorescent marker.

For the determination of the ultrafast response of the fluorescent label, all samples were excited either at 540 and 560 nm. Probe wavelengths ranging from 560 to 600 nm were used. The results obtained with an excitation wavelength of 540 nm are shown in Figure 2. A biexponential decay function was fitted to all data:

\[
\text{OD}(t) = y_0 + A_1 \cdot e^{-t/\tau_1} + A_2 \cdot e^{-t/\tau_2}.
\] (1)

Here, OD(t) is the normalized, observed transient absorbance; \( y_0 \) is a constant offset; and \( A_1, A_2 \), and \( \tau_1, \tau_2 \) are the amplitudes and time constants of a fast and slow decaying component of the biexponential fit. For a more detailed analysis of ultrafast decays, more than 2 exponentials might be appropriate. However, in the present analysis of a proteolytic cleavage, a biexponential fit is sufficient. The corresponding fitting parameters are given in Table 1. The relative amplitudes, \( A_1 \) and \( A_2 \), of the fast and slow components of the response correlate well with the size of the residual fragment attached to the fluorescent label. In addition, there is a correlation in the time constant of the slow component, \( \tau_2 \), with the fragment size, except for the pure dye in buffer solution. Because the 2 timescales \( \tau_1 \) and \( \tau_2 \) are quite different, we repeated the fitting procedure with a common, globally fitted value for \( \tau_2 \) for all samples to determine the values of \( \tau_1 \) more accurately. This procedure still produces quite reasonable fits. The corresponding fitting results are given in the right-hand side of Table 1 and show that the \( \tau_1 \) value also corresponds very well with the fragment size. In Figure 3, all parameters are plotted as a function of the average molecular mass of the residual fragment size.

Similar results can also be obtained with an excitation wavelength of 560 nm and detection wavelengths ranging from 580 to 600 nm (data not shown). The general trend is very similar except that \( \tau_1 \) and \( \tau_2 \) are significantly faster, and the amplitude \( A_1 \) becomes negative for a detection wavelength of 600 nm. Furthermore, some of the ultrafast decays obtained using this detection wavelength could not be described well with a biexponential function.

DISCUSSION

In the first section of the discussion, we focus on the applicability of ultrafast spectroscopy to monitor biomolecular reactions fast enough to enable HTS. The second section treats theoretical aspects of the mechanisms by which biochemical reactions influence the ultrafast response of reporter molecules and biomolecules.

Ultrafast parameters suitable for HTS

One purpose of the present investigation was to elucidate whether the ultrafast response of marker molecules or biomolecules themselves can be used for fast and efficient observation of biochemical reactions. The most dominant effect of the proteolytic cleavage on the decay kinetics is a drastically changing ratio of the amplitudes of the ultrafast decay (< 10 psec) and the decay on the psec timescale (~150–250 psec) \( A_1/A_2 \) (Table 1). In addition, the time constant of the faster decay component, \( \tau_1 \), reflects very well the change in the fluorophore environment. The acquisition of ultrafast decay curves, such as shown in Figure 2, usually requires about 5 min because each point of the single pump and probe beam delay has to be measured separately. For a fast biochemical analysis, we decided to use only 2 of these time points to achieve measuring times of less than or equal to 1 sec. We tested various measurement parameters such as excitation and probe wavelengths, \( \lambda_{\text{pump}} \) and \( \lambda_{\text{probe}} \), as well as different pump probe delays, \( t \), and detection windows. The set of parameters we found to be best for monitoring the fragment size is the ratio of the ultrafast response measured at 50 psec divided by the response measured at 0 psec, observed using an excitation wavelength of \( \lambda_{\text{pump}} = 540 \) nm and a probe wavelength of \( \lambda_{\text{probe}} = 570 \) nm. This ratio is subject to a large change for differing protein fragment masses, and it displayed
a small experimental error in repetitive measurements. The semilogarithmic plot in Figure 4 demonstrates that this parameter can serve as a robust parameter for the molecular mass over several orders of magnitude.

Such a ratiometric detection has the same advantages as other ratiometric detection schemes such as fluorescence anisotropy or 2-color fluorescence detection, which do not depend on variations in the concentration of the reporter molecule or instrumental
### Table 1. Biexponential Fitting Parameters for the Data Presented in Figure 2 (Left) and Corresponding Parameters Obtained with Globally Fitted \( \tau \) Values (Right)

<table>
<thead>
<tr>
<th>( \lambda_{\text{pump}} ) nm</th>
<th>TMR</th>
<th>TMR-BSA-Subtilisin</th>
<th>TMR-BSA-Elastase</th>
<th>TMR-BSA</th>
<th>( \lambda_{\text{pump}} ) nm, Globally Fitted</th>
<th>TMR</th>
<th>TMR-BSA-Subtilisin</th>
<th>TMR-BSA-Elastase</th>
<th>TMR-BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>580</td>
<td>( \tau_1 ) psec</td>
<td>4.57 ± 1.81</td>
<td>2.69 ± 0.9</td>
<td>6.89 ± 0.56</td>
<td>3.18 ± 0.27</td>
<td>( \tau_1 ) psec</td>
<td>2.7 ± 1.10</td>
<td>6.2 ± 1.08</td>
<td>6.8 ± 1.10</td>
</tr>
<tr>
<td></td>
<td>( \tau_2 ) psec</td>
<td>201 ± 20</td>
<td>164 ± 3</td>
<td>126 ± 2</td>
<td>94 ± 1</td>
<td>( \tau_2 ) psec</td>
<td>120</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>( A_1 )</td>
<td>0.016 ± 0.27</td>
<td>0.098 ± 0.017</td>
<td>0.237 ± 0.009</td>
<td>0.223 ± 0.009</td>
<td>( A_1 )</td>
<td>0.063 ± 0.12</td>
<td>0.22 ± 0.02</td>
<td>0.27 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>( A_2 )</td>
<td>1.08 ± 0.03</td>
<td>0.877 ± 0.007</td>
<td>0.721 ± 0.007</td>
<td>0.763 ± 0.005</td>
<td>( A_2 )</td>
<td>0.94 ± 0.01</td>
<td>0.74 ± 0.01</td>
<td>0.68 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>( A_1/A_2 )</td>
<td>67.5</td>
<td>8.98</td>
<td>3.04</td>
<td>3.42</td>
<td>( A_1/A_2 )</td>
<td>14.8</td>
<td>3.3</td>
<td>2.47</td>
</tr>
<tr>
<td>570</td>
<td>( \tau_1 ) psec</td>
<td>1.41 ± 0.39</td>
<td>5.81 ± 0.5</td>
<td>5.89 ± 0.4</td>
<td>5.86 ± 0.3</td>
<td>( \tau_1 ) psec</td>
<td>1.6 ± 0.4</td>
<td>1.9 ± 0.6</td>
<td>8.24 ± 0.55</td>
</tr>
<tr>
<td></td>
<td>( \tau_2 ) psec</td>
<td>166 ± 2</td>
<td>248 ± 4</td>
<td>144 ± 3</td>
<td>122 ± 2</td>
<td>( \tau_2 ) psec</td>
<td>170</td>
<td>170</td>
<td>170</td>
</tr>
<tr>
<td></td>
<td>( A_1 )</td>
<td>0.101 ± 0.016</td>
<td>0.218 ± 0.01</td>
<td>0.255 ± 0.009</td>
<td>0.362 ± 0.01</td>
<td>( A_1 )</td>
<td>0.104 ± 0.015</td>
<td>0.173 ± 0.031</td>
<td>0.29 ± 0.009</td>
</tr>
<tr>
<td></td>
<td>( A_2 )</td>
<td>0.906 ± 0.004</td>
<td>0.738 ± 0.005</td>
<td>0.696 ± 0.006</td>
<td>0.608 ± 0.059</td>
<td>( A_2 )</td>
<td>0.901 ± 0.003</td>
<td>0.83 ± 0.007</td>
<td>0.647 ± 0.005</td>
</tr>
<tr>
<td></td>
<td>( A_1/A_2 )</td>
<td>9.01</td>
<td>3.39</td>
<td>2.73</td>
<td>1.68</td>
<td>( A_1/A_2 )</td>
<td>8.7</td>
<td>4.74</td>
<td>2.23</td>
</tr>
<tr>
<td>560</td>
<td>( \tau_1 ) psec</td>
<td>1.58 ± 0.3</td>
<td>7.74 ± 2.26</td>
<td>6.82 ± 1.47</td>
<td>7.19 ± 0.3</td>
<td>( \tau_1 ) psec</td>
<td>3.45 ± 1.49</td>
<td>1.99 ± 2.35</td>
<td>7.37 ± 1.83</td>
</tr>
<tr>
<td></td>
<td>( \tau_2 ) psec</td>
<td>173 ± 2</td>
<td>270 ± 7</td>
<td>215 ± 8</td>
<td>144 ± 3</td>
<td>( \tau_2 ) psec</td>
<td>201</td>
<td>201</td>
<td>201</td>
</tr>
<tr>
<td></td>
<td>( A_1 )</td>
<td>0.135 ± 0.014</td>
<td>0.124 ± 0.016</td>
<td>0.234 ± 0.018</td>
<td>0.34 ± 0.009</td>
<td>( A_1 )</td>
<td>0.146 ± 0.036</td>
<td>0.137 ± 0.106</td>
<td>0.221 ± 0.027</td>
</tr>
<tr>
<td></td>
<td>( A_2 )</td>
<td>0.839 ± 0.003</td>
<td>0.763 ± 0.01</td>
<td>0.673 ± 0.013</td>
<td>0.591 ± 0.006</td>
<td>( A_2 )</td>
<td>0.805 ± 0.011</td>
<td>0.838 ± 0.02</td>
<td>0.691 ± 0.128</td>
</tr>
<tr>
<td></td>
<td>( A_1/A_2 )</td>
<td>6.2</td>
<td>6.18</td>
<td>2.9</td>
<td>1.74</td>
<td>( A_1/A_2 )</td>
<td>5.52</td>
<td>6.12</td>
<td>3.13</td>
</tr>
</tbody>
</table>

The given errors represent the goodness of the fit. Statistical errors representing the uncertainty of the ultrafast readout for a biochemical analysis are given in Table 2 and in Figures 4 and 5. TMR, tetramethylrhodamine; BSA, bovine serum albumin.

a. For these data, a biexponential fitting procedure did not result in robust parameters.

### Theoretical aspects of the ultrafast response

The question arises about what changes in the biomolecular environment of an excited molecule contribute to the pump probe signals and to what extent other biochemical reactions, such as receptor-ligand binding or other enzymatic reactions, will affect the ultrafast response of reporter or biomolecules. In parameters. The histogram presented in Figure 5 demonstrates that the chosen set of parameters is also capable of determining the proteolytic progress within a measuring time of 600 msec. This measuring time included the successive determination of the transient absorption signals at 0 psec and 50 psec, each requiring 300 msec. In principle, the measuring time can be reduced to 300 msec by using a duplicated detection scheme measuring the responses at 0 and 50 psec simultaneously.

The accuracy of an assay readout is often determined by the Z’ factor:

\[
Z' = 1 - 3 \frac{\sigma_+ + \sigma_-}{|\bar{X}_+ - \bar{X}_-|}.
\]

Here, \( \bar{X}_+ \) and \( \bar{X}_- \) are the mean values of a parameter of the positive and negative sample, and \( \sigma_+ \) and \( \sigma_- \) are the corresponding standard deviations. The Z’ factor cannot exceed the value 1, which corresponds to the best possible assay readout quality. Z’ factors above 0.5 are usually regarded accurate enough. With the smallest investigated sample (TMR as positive control and untreated BSA as negative control), we obtain an instrumental Z’ factor of 0.83 for a measurement time of 1 sec. A 1-sec measurement time corresponds to about 88,000 samples per day, which is close to the limit of 100,000, which is usually regarded as the lower limit of ultra-HTS. The Z’ factor determined for a 600-msec measurement time (144,000 samples per day) is 0.64. We also tested other sets of parameters, such as a pump probe delay of 80 psec or a probing wavelength of 580 nm. The resulting Z’ factors are shown in the upper part of Table 2. In principle, it is feasible to combine different probing wave-lengths in 1 measurement of the same duration, which gives the potential to further enhance the assay readout, reduce the measurement time, or give more specific information about the biochemical reaction. We also tested the stability of the assay readout with respect to measurements performed at different days. We obtained a day-to-day Z’ factor for different samples of 0.77 (Table 2, lower part). Commonly, sophisticated handling and laser equipment are associated with ultrafast measurements. Our results show that the development of modern ultrafast laser equipment allows us to use the ultrafast response as a robust and fast analytical tool for HTS (>10,000 samples a day) or even ultra-HTS (>100,000 samples a day).
the following, we give a brief insight into energy relaxation processes and solvation dynamics contributing to the ultrafast response either of reporter molecules or the biomolecules themselves.

Among the fastest processes usually observed after the excitation of a molecule by an ultrashort pump pulse is IVR, which usually takes place on a subpicosecond timescale. It is controlled by the coupling between different vibrational modes of the excited molecule itself. Thus, changes in the biomolecular environment usually affect the IVR processes only to a smaller extent. On a timescale of about 1 to 50 psec, the excited molecule is influenced by the VET or “vibrational cooling” to the solvent or the protein environment (yellow arrows in Fig. 6). The extent and velocity of this process depends on the vibrational...

**FIG. 3.** Biexponential fitting parameters plotted as a function of molecular mass (semilogarithmic plot). $\lambda_{\text{pump}} = 540$ nm. $\lambda_{\text{probe}} =$ (a) 580 nm, (b) 570 nm, and (c) 560 nm, respectively.
coupling between the excited molecule and its nearest environment, as well as the amount of excess energy introduced by the pump pulse. A pulsed excitation of the solute molecule in a polar solvent also leads to the instantaneous change of the molecule’s dipole (i.e., an instantaneous electric field affects the molecule’s environment). When molecules in the environment of the excited molecule have a significant permanent dipole moment, a rearrangement to a new equilibrium position by rotational and translational motions occurs (red arrows in Fig. 6). As this process carries on, the energy of the excited molecule is lowered, resulting in a red shift in the fluorescence spectrum, the dynamic stokes shift.

Both processes, VET and the reorientation in the excited molecule’s environment, similarly affect the transient absorption of the excited molecule and potentially induce emission from the excited molecule, which both can contribute to the signal detected by the probe pulse at a distinct wavelength.

An aqueous solvent environment is typically discussed in terms of solvation shells. The first solvation shell represents water molecules in the direct neighborhood (up to ~7 Å; see Fig. 6) of the excited molecule, which are to some extent bound to the excited molecule or the covalently linked protein. After an excitation, the pump probe signals are influenced by rotational and slightly by translational motions of these nearest neighboring molecules of the first solvation shell.

More remote solvent molecules are often referred to as bulk water. In bulk water, the relaxation includes high-frequency vibration (hindered rotations) and intermolecular vibration.  

Table 2. Z Factors of the Ultrafast Assay Readout Obtained for Different Pump Probe Delays and Probing Wavelengths (Top) and Ratiometric Detection Response (80 psec Divided 0 psec) Obtained for Different Samples at Different Measurement Days with Excitation at 540 nm and Detection at 570 nm (Bottom)

<table>
<thead>
<tr>
<th>Pump Probe Delays, t, Used for Ratiometric Analysis</th>
<th>Measuring Time</th>
<th>Excitation Wavelength</th>
<th>Detection Wavelength</th>
<th>Z' Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 psec and 0 psec</td>
<td>1 sec</td>
<td>540 nm</td>
<td>570 nm</td>
<td>0.83</td>
</tr>
<tr>
<td>80 psec and 0 psec</td>
<td>1 sec</td>
<td>540 nm</td>
<td>570 nm</td>
<td>0.675</td>
</tr>
<tr>
<td>100 psec and 0 psec</td>
<td>1 sec</td>
<td>560 nm</td>
<td>580 nm</td>
<td>0.22</td>
</tr>
<tr>
<td>50 psec and 0 psec</td>
<td>600 msec</td>
<td>540 nm</td>
<td>570 nm</td>
<td>0.64</td>
</tr>
<tr>
<td>80 psec and 0 psec</td>
<td>600 msec</td>
<td>540 nm</td>
<td>570 nm</td>
<td>0.53</td>
</tr>
<tr>
<td>TMR-BSA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>0.384 ± 0.017</td>
<td>0.45 ± 0.016</td>
<td>0.544 ± 0.034</td>
<td>0.625 ± 0.038</td>
</tr>
<tr>
<td>Day 2</td>
<td>0.376 ± 0.045</td>
<td>0.42 ± 0.011</td>
<td>0.55 ± 0.009</td>
<td>0.709 ± 0.018</td>
</tr>
</tbody>
</table>

TMR, tetramethylrhodamine; BSA, bovine serum albumin.
FIG. 6. Schematic presentation of processes contributing to the ultrafast response of a fluorescence label attached to proteins. Yellow arrows indicate vibrational energy transfer; red arrows indicate protein and solvent reorientation. Shorter arrows refer to slower processes.
Both water molecules in the first solvation shell and bulk water contribute to the pump probe signal on very different timescales. This difference in timescales enables a distinction of the response in the nearest and remote environments of the excited molecule. Experiments done with extrinsic fluorescent probes such as coumarin or eosin labeled to biomolecules showed multiphasic behavior reflecting a convolution of the responses of the protein, the water in the hydration shell, and the bulk water. It is known from such experiments and theoretical studies that in general, the VET, the dynamics in the bulk water shell, the first solvation shell, and the protein relaxation dynamics have timescales ranging from less than 1 psec to ~300 psec. All of these processes contribute to the observed pump probe signals at different timescales and different excitation and detection wavelengths and can potentially serve as an indicator for a distinct change in the biomolecular environment.

The most obvious correlation with the proteolytic cleavage in the present data is an increasing amplitude of the faster decay component, A1 (1-10 psec), and a corresponding decreasing amplitude of the slower decay component, A2 (250-350 psec), with increasing fragment sizes. The ratio A2/A1 is up to 1 order of magnitude larger for the free-label TMR in comparison to TMR-labeled BSA (Table 1). This alternation in the value of this ratio is likely the result of different contributions from the bulk water and the first solvation shell response, as well as from the protein relaxation and VET for different sizes of protein fragments. For other biochemical reactions, such as receptor-ligand binding, probably the induced changes in the first solvation shell or in the VET to the environment are suitable to monitor the progress of the reaction. For instance, the significant change in the time constant of the fast decay component in the present example (τ1, Fig. 3b) could reflect different timescales for VET associated with changes in the nearest biochemical environment. In this context, other ultrafast technologies, such as 2-dimensional vibrational spectroscopy, which depend on vibrational couplings between molecules, are also expected to display specific interactions probably even between smaller molecules or proteins without any fluorescence label. The feasibility of using such approaches to monitor other biochemical reactions will be the subject of future studies.

**CONCLUSIONS**

In the present report, it has been shown that the ultrafast response of a reporter molecule is well suited to monitor biochemical reactions within only a few hundreds of milliseconds of measurement time. The ratio of the amplitude of the transient absorption (λ\text{trans} = 540 nm, λ\text{probe} = 570 nm), observed at the 50-psec and 0-psec pump probe delay, can be used as a robust parameter for monitoring the progress of a proteolytic cleavage. The Z’ factor of the readout of the presented example is 0.83 and 0.64, with a measurement time of about 1 sec per sample (Fig. 4) and 600 msec per sample (Fig. 5), respectively. This corresponds to a very good quality of the assay readout, which is sufficient for HTS or even ultra-HTS applications.

The most obvious correlation in the present data with the proteolytic cleavage is an increasing amplitude of the faster decay component, A1 (1-10 psec), and a corresponding decreasing amplitude of the slower decay component, A2 (250-350 psec), with increasing fragment sizes. In addition, the time constant of the faster decay component, τ1, increased with the size of the protein fragments. We assign the dominant part of the observed changes during the proteolytic cleavage to changing contributions from fast bulk water relaxation and differing timescales of protein and bulk water reorientation. In addition, the timescales of the fast vibrational cooling into the water or protein environment display changes in the nearest biochemical environment of the fluorescence marker.

The intrinsically restricted detection volume of typical ultrafast detection schemes will allow measurements in microscopic dimensions. Modern ultrafast laser systems are stable enough for industrial environments, and the sensitivity of ultrafast detection can easily be improved by simple modifications such as the use of more than 1 detector. The multitude of known ultrafast detection schemes and the complex ultrafast response of reporter molecules have the potential to monitor even more sophisticated biochemical reactions such as receptor-ligand binding or other enzymatic reactions. In addition, the measurement of biomolecules without a fluorescence label could become feasible because the ultrafast response can, in principle, be observed with any molecule showing a significant absorption in its ground and/or excited states.

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**REFERENCES**


