DOI: 10.1002/cphc.200800586

Two-Color Two-Photon Excitation of Intrinsic Protein Fluorescence: Label-Free Observation of Proteolytic Digestion of Bovine Serum Albumin

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Two-color two-photon (2c2p) excitation fluorescence is used to monitor the enzymatic cleavage of bovine serum albumin (BSA) by subtilisin. Fluorescence is generated by irradiation with spatially and temporally overlapping femtosecond laser beams resulting in simultaneous absorption of an 800 and a 400 nm photon. Thereby, excitation of the fluorescent amino acid tryptophan present in BSA corresponds to an effective one-photon wavelength of 266 nm. The progress of protein cleavage is monitored by time-resolved fluorescence analysis. The fluorescence lifetime of tryptophan decreases during the reaction. This demonstrates a novel label-free multiphoton observation technique for conformational changes of proteins containing tryptophan. Due to the strong 2c2p fluorescence signal it is suitable for fast evaluation and monitoring of protein reactions. The course of the reaction is monitored simultaneously by gel electrophoresis. In contrast to conventional one-photon techniques, 2c2p excitation enables label-free protein fluorescence studies without irradiating the sample with UV light. Due to the dependence of the excitation on the power of both laser beams, excitation is limited to a relatively small focal volume. This results in dramatically reduced overall photodamage compared to direct UV irradiation. This method can be easily extended to microscopy imaging techniques.

1. Introduction

Fluorescence assays have become an indispensable tool for biologically relevant research.[1] For that reason very sophisticated labeling techniques have been developed to match almost every requirement.[2] Additionally, a vast arsenal of differently labeled biomolecules is available ready for use. Although labeling of biomolecules offers many possibilities and advantages, it is not the undisturbed biological system that is under observation. Since typical fluorogenic groups introduced into biomolecules are not very small, the system may behave differently. Furthermore, from a practical point of view, labeling requires an additional step of preparation, which may be easy for simple biochemical reactions but can be challenging for in vivo experiments. This problem can be avoided by using intrinsic fluorescence. Especially in the case of proteins, staining is often not necessary, because many proteins contain fluorescent amino acids[1] which can function as a natural fluorescence tag that allow the undisturbed biological system to be monitored.

Of the three fluorescent amino acids (phenylalanine, tyrosine, and tryptophan), tryptophan offers the highest fluorescence quantum yield.[1] Consequently, it is widely used for all kinds of autofluorescence assays, as well as for everyday applications such as protein detection in HPLC and gel electrophoresis.

The main drawback for all these applications is the fact that the absorption maximum of tryptophan (280 nm)[1] and the other fluorescent amino acids lies in the UV, where many biological compounds are sensitive. Photostress to DNA is especially undesirable.[3] Hence, strong irradiation with UV light can photodamage the system under investigation.[4,5] For microscopy applications, the use of one-photon excitation in the UV region is additionally limited by increased scattering and the fact that conventional microscope optics do not transmit at the desired wavelengths. Two-photon techniques circumvent these problems.[6] Due to the quadratic dependence of absorption on excitation power, excitation is limited to the focal region, in case of a microscope objective to a very small volume.[7] This reduction of excitation volume effectively reduces the overall photodamage. However, as two-photon absorption cross sections are extremely small,[8] very high power levels are required to achieve efficient excitation. These high power levels can only be applied to biological samples by using pulsed excitation.[9] Otherwise, the introduced amount of energy would be too high. Thus, laser pulses should be as short as possible. Currently, the ideal excitation source for two-photon excitation is the Ti:sapphire (Ti:Sa) femtosecond laser providing ultrashort laser pulses with extremely low pulse energies.[10] Unfortunately, femtosecond laser sources which provide sufficient power for two-photon excitation are still limited to the red or near-IR region (700–1000 nm) offering an effec-

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tive spectral window for two-photon excitation from 350 to 500 nm.

One possibility to overcome this restriction in wavelength is the use of two photons of different color. Using the fundamental and second harmonic of a Ti:Sa laser results in two-color, two-photon (2c2p) absorption equivalent to an effective excitation wavelength between 233 and 333 nm.[11,12] For example, two spatially and temporally overlapping laser pulses of 400 and 800 nm can be used to excite tryptophan or other UV fluorophores at an effective excitation wavelength of 266 nm. Therefore, 2c2p excitation allows highly time resolved fluorescence lifetime measurements without applying UV light to the sample. Previously, we showed experimentally that 2c2p excitation of different chromophores using spatially and temporally overlapping femtosecond laser pulses is an excellent alternative to one-photon excitation and a useful extension to conventional two-photon techniques.[12]

Analogously to conventional one-color two-photon excitation, excitation is limited to the focal region due to the quadratic dependence on the total excitation power, or, to be more precise, on the product of the powers of the two colors. In addition to the resulting intrinsic 3D resolution, which is important for microscopy applications, this offers the possibility of altering the power ratio of the two colors independently without changing the generated fluorescence intensity. For example, using excess power at 800 nm can be useful if the sample is potentially sensitive to high intensities at 400 nm. Furthermore, 800 nm light is scattered less than 400 nm light by most biological media. Therefore, higher penetration depths can be achieved.

Along with this newly gained access to multiphoton excitation of tryptophan and other UV fluorophores the 2c2p technique offers an excellent basis for time-resolved fluorescence measurements such as fluorescence lifetime and fluorescence anisotropy decay. The introduction of femtosecond laser pulses allowed the use of all known time-domain techniques, such as time gating and time-correlated single-photon counting (TCSPC), which results in excellent time resolution.[13,14] Furthermore, the fluorescence lifetime of tryptophan is very sensitive to its surroundings.[15–18] Thus, the combination of multiphoton excitation and time-resolved measurement of fluorescence offers a multitude of applications in monitoring protein reactions or modifications.

Measurement of fluorescence lifetimes is a powerful tool giving significant additional information compared to pure intensity measurements.[1,19,20] Measurements of fluorescence lifetimes are independent of concentration and excitation intensity. Thus, this method is relatively insensitive to uneven distribution of the fluorophore or fluctuation in laser power.

Fluorescence lifetime is strongly influenced by a large variety of different interactions between the fluorophore and its surroundings. Specifically designed fluorophores will change their fluorescence lifetime when a specific parameter of the environment changes. Therefore, highly specialized probes for all kinds of parameters have been established.[1,21–24] With the appropriate excitation source, fluorescence lifetime measurements of autofluorescence from biological samples can be used to monitor changes in the environment of the respective fluorophore. Although the fluorescence lifetime of these endogenous fluorophores is often much more complicated to interpret, it can provide valuable information about processes inside a biological system (e.g., binding of NADPH to enzymes).[25,26]

To the best of our knowledge, we present the first 2c2p-based protein fluorescence assay and present combined application of 2c2p excitation and fluorescence lifetime measurements. As an example we monitored the progress of the proteolytic cleavage of bovine serum albumin (BSA) by subtilisin.[27]

The strong fluorescence signal allows short acquisition times even with a rather simple experimental setup. A more sophisticated setup using high numerical aperture objectives will enhance the efficiency of excitation as well as the fluorescence detection, and thus allow bioassays fast enough for high-throughput screening.

2. Results and Discussion

We studied 2c2p excitation fluorescence of tryptophan and the protein bovine serum albumin (BSA), which contains three tryptophan residues. Two-color two-photon detection of tryptophan in solutions has been studied previously using femtosecond laser pulses.[12] Here we present the first application of 2c2p excitation to intrinsic protein fluorescence.

2.1. Cross-Correlation Experiment

The experimental setup for 2c2p excitation fluorescence with femtosecond laser pulses is shown in Figure 1. For realization of 2c2p excitation temporal and spatial overlap of the femtosecond pulses is required. Figure 2 illustrates the data obtained from a cross-correlation experiment in which the delay between the two excitation laser pulses was altered by using the optical delay line implemented in the optical path of the red light.

As expected, 2c2p fluorescence from tryptophan occurs only when both laser pulses coincide at the same time in the

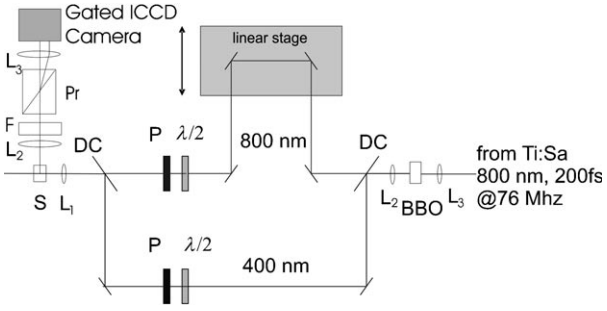

Figure 1. Experimental setup for 2c2p excitation fluorescence using femtosecond laser pulses. BBO = barium borate crystal for frequency doubling, L = lens, DC = dicroic mirror, $\lambda/2$ = half-wave plate, F = set of filters (Semrock NF01-405U and Semrock NF01-808U, both at 10° angle, and a interference filter 340/80 nm), and Pr = Rochon quartz prism, 5° deviation angle. The linear stage is used to adjust temporal overlap of the pulses in the sample.

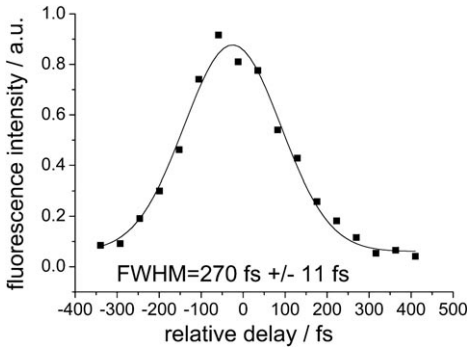

Figure 2. Cross-correlation experiment with a 0.1 mM solution of BSA in PBS. Maximum fluorescence intensity is observed for no time delay between the pulses. With increasing delay the fluorescence intensity decreases. A Gaussian distribution is fitted to the experimental data.

sample. Lack of temporal overlap of the pulses leads to a fluorescence signal of less than 2% of that obtained from overlapping excitation pulses. The solid curve in Figure 2 is a Gaussian distribution fitted to the experimental data by using a nonlinear least-squares fitting procedure. The full width at half-maximum (FWHM) of the cross-correlation curve of 272 fs is in good agreement with the initial pulse width of about 200 fs.

2.2. 2c2p Excitation of Intrinsic Protein Fluorescence

Figure 3 shows the fluorescence signals from a 0.1 mM solution of BSA in phosphate buffer solution (PBS). The columns labeled with "400 nm + 800 nm" represent the signal on irradiation of

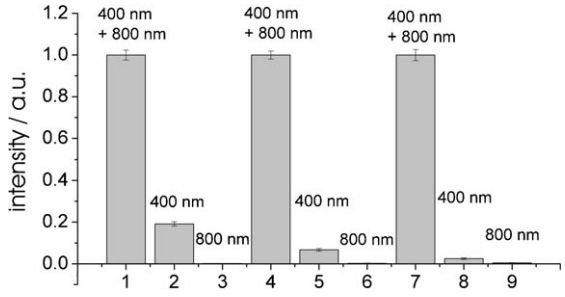

Figure 3. Fluorescence intensities on irradiation of a 0.1 mM BSA solution in PBS with 400 and 800 nm light at different power levels. Columns labeled with "400 nm + 800 nm" represent the 2c2p excitation fluorescence signal which is obtained by subtracting the fluorescence signal resulting from irradiation with one color only from the total fluorescence signal. For better comparison the data are normalized to the 2c2p fluorescence signal. Error bars indicate the standard deviation of 30 measurements. Power levels: columns 1–3: 800 nm: 112.5 mW, 400 nm: 8.0 mW; columns 4–6: 800 nm: 225 mW, 400 nm 4 mW; columns 7–9: 800 nm: 450 mW, 400 nm: 2.0 mW.

both colors, and those labeled with either "400 nm" or "800 nm" the signal on irradiation with one color. The strongest fluorescence signal occurs when the 400 and 800 nm beams coincide in the sample. As long as the product of the excitation powers of both colors remain constant, variation of the excitation power leads to a constant fluorescence signal, provided that the fluorescence signal of illumination with each color alone is subtracted from the total fluorescence. For illumination at 800 nm almost no signal can be detected for all applied power levels. Hence, no three-photon excitation is observed. Excitation at 400 nm leads to a weak fluorescence signal. The dependence of the fluorescence signal for irradiation exclusively at 400 nm is shown in Figure 4. This is consistent with 1c2p excitation of tryptophan at the strong band around 200 nm in its absorption spectrum.[1] For such a 1c2p excitation a quadratic dependence of the fluorescence signal on the excitation power is expected. The presence of such a two-photon process is confirmed by the slope in Figure 4. A logarithmic plot leads to a slope of 1.7. Although smaller than two, this value clearly indicates a two-photon process as the origin of the fluorescence signal. The deviation could be caused by a linear background signal or may be explained by intramolecular energy transfer out of the excited S_2 state.

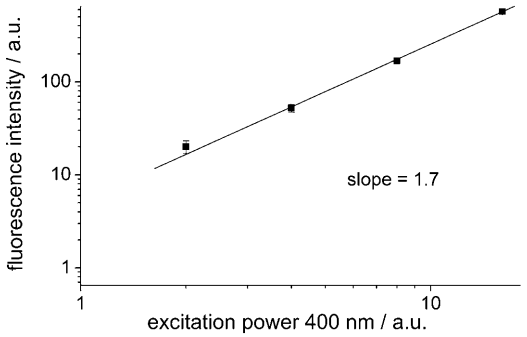

Figure 4. Dependence of the fluorescence signal on excitation intensity on irradiation at 400 nm exclusively. The slope indicates a two-photon process with small linear contribution.

However, as demonstrated in Figure 3, this background signal resulting from 1c2p excitation at 400 nm can be almost completely suppressed by decreasing the power at that wavelength. The second column from the right represents a background signal from 400 nm irradiation of about 2% of the 2c2p signal. The loss in 2c2p fluorescence signal is compensated by increasing the power at 800 nm. This is possible because the fluorescence intensity is dependent on the product of the photon fluxes of the two colors [Eq. (1)]

$$dN/dt = -\delta I_1(800\ \mathrm{nm}) I_2(400\ \mathrm{nm}) N$$

where N is the number of molecules in the ground state, δ the two-photon absorption cross section, and I the photon flux.

Hence, excess intensity at 800 nm can be used while only low intensities at 400 nm are necessary. This has a significant positive effect when using 2c2p excitation in microscopy. Near-infrared light can penetrate much deeper into biological samples as it is much less scattered or absorbed than visible or UV light. This allows a high penetration depth of the imaging method and also keeps photodamage low.

2.3. Fluorescence Lifetime after 2c2p Excitation

After adjustment of the excitation intensities for the optimal 2c2p excitation fluorescence signal, fluorescence lifetime measurements in the time domain were performed. The fluorescence lifetimes of tryptophan and BSA are shown in Figure 5. The lifetime of tryptophan resulting from a monoexponential fit of 2.5 ns is consistent with observations from Fleming et al. for one-photon excitation.[16] Monoexponential fit of the fluorescence intensity decay of BSA gave a fluorescence lifetime of 6.1 ns which, again agrees with fluorescence lifetime measurements performed with one-photon and 1c2p excitation.[28] Consequently we can conclude that the fluorescence occurs from the same excited stated, that is, the S_1 state.

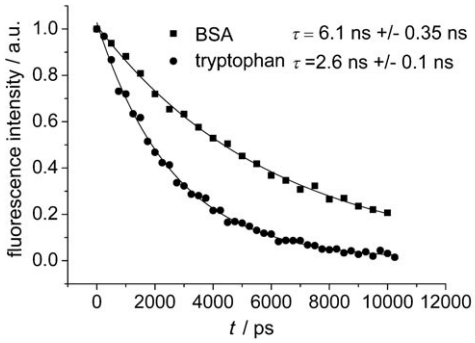

Figure 5. Fluorescence lifetimes of 0.1 mM solutions of tryptophan and BSA in PBS. Mono-exponential fitting gives (mean) fluorescence lifetimes of 2.6 ns for tryptophan and 6.1 ns for BSA.

solution without BSA gave a fluorescence intensity of only 6% of the intensities obtained at the beginning of the observation of the 0.1 mM BSA enzyme reaction. A mono-exponential decay was found to be the overall best fit for the intensity decay data. The obtained fluorescence lifetime decreases with increasing reaction time. Shortly after the start of the enzymatic reaction, the fluorescence lifetime drops from its initial value of about 6 ns to 4.5 ns. With ongoing cleavage the lifetime decreases further before it settles at a final value of less than 2 ns. The decay of fluorescence lifetime during the reaction is illustrated by the plots of lifetime versus reaction time shown in Figures 6C and D. After the initial fast drop in lifetime, a mono-exponential fit describes the data well. For the reaction with the higher BSA concentration a decay constant of 404 ± 23 min was obtained. For the lower concentration a value of 59 ± 4 min was found.

The correlation of the fluorescence lifetime shown in Figure 6 with the results of gel electrophoresis (see below) leads to the conclusion that the fluorescence lifetime of the intrinsic protein fluorescence decreases with decreasing fragment size. This is somewhat surprising, since it is known that the fluorescence lifetime of tryptophan is dependent on the conformation of the protein in a very complicated way and it eventually depends also on the conformation of tryptophan itself.[18,29] The rotational conformations of tryptophan itself as well as the quenching ability of its neighborhood in most cases have a far bigger effect on its lifetime than the overall size or weight of the protein. Hence, it can be concluded that in BSA the tryptophan residues are in a conformational state for which the rate constants for quenching processes are rather slow. The quenching rate obviously increases when the

2.4. Label-Free Monitoring of Protein Digestion

Proteolytic digestion of BSA by the hydrolase subtilisin was monitored by means of fluorescence lifetimes after 2c2p excitation of intrinsic fluorescence of BSA. It was found that degradation of BSA into smaller fragments by the protease subtilisin leads to a significant change in fluorescence lifetime. Figure 6 shows the decay of fluorescence intensity at arbitrarily selected times of the enzymatic reaction. The data of Figure 6A were obtained with a 1 mM solution of BSA in PBS, while those of Figure 6B were obtained from a 0.1 mM solution of BSA in PBS. Subtilisin (0.01 mM) was added to each protein solution. Although subtilisin also contains tryptophan, a 0.01 mM enzyme

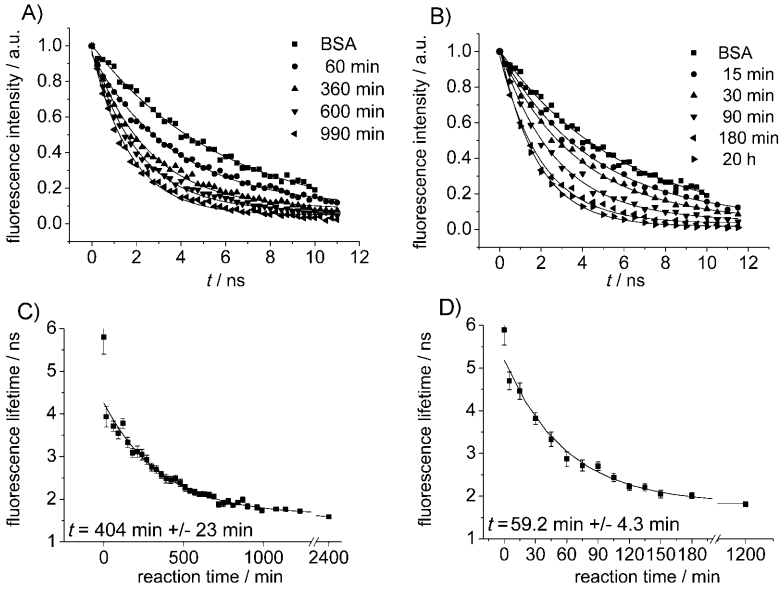

Figure 6. Selected fluorescence decays at different times of the reaction (A, B). A) Data from a 1 mM BSA solution in PBS. B) Data from a 0.1 mM BSA solution in PBS. To either solution about 0.01 mM subtilisin was added. Solid lines represent monoexponential fitting for determination of the fluorescence lifetime. In C) and D) the obtained fluorescence lifetimes are plotted versus the reaction time. After a rapid decay of the fluorescence lifetime during the first reaction period, a mono-exponential decay fits the data best.

fragments become smaller, and a reduced fluorescence lifetime results.

It is noteworthy that the final fluorescence lifetime settles below the fluorescence lifetime of free tryptophan. This is consistent with the experiments performed by Fleming et al.,[16] who measured a fluorescence lifetime for a dipeptide of tryptophan of about 1.5 ns. Hence, the measured lifetime indicates that at the end of the reaction polypeptides are still present. If the proteolytic cleavage proceeded to a stage of isolated amino acids a longer fluorescence lifetime around 2.5 ns is expected.

The data presented in Figure 6 demonstrate the possibility of label-free monitoring of the proteolytic cleavage of BSA by subtilisin by means of fluorescence lifetime measurements of the intrinsic protein fluorescence. It is surprising that a monoexponential decay is the best fit to the intensity decay. The fluorescence of a single tryptophan protein like human serum albumin is usually described as a bi- or even triexponential decay.[30] By comparison with the results from gel electrophoresis it can be assumed that the measured intensity decays represent a superposition of a relatively broad distribution of different lifetimes arising from a broad distribution of protein fragments with different molecular weights and conformations. Nevertheless, applying a stretched exponential to the data does not improve the fit.[31]

2.5. Fluorescence Anisotropy

To ensure that the measurements of the fluorescence lifetime are not significantly influenced by anisotropy effects, the fluorescence anisotropy decay of BSA was measured. Probably due to internal energy transfer, the fluorescence of tryptophan in BSA exhibits a very small initial fluorescence anisotropy of 0.07. This very low anisotropy does not allow determination of the rotational diffusion constant with acceptable accuracy. Hence, the rotational diffusion, which should decrease with increasing size of the protein fragments cannot be measured with sufficient accuracy. However, it clearly demonstrates that the fluorescence lifetime measurements are not disturbed by anisotropy effects.

2.6. Gel Electrophoresis

The fluorescence decay measurements of the proteolytic cleavage of BSA were complemented by monitoring the reaction by gel electrophoresis. Figure 7 shows the gel for a 1 mM BSA solution in PBS corresponding to the fluorescence data presented in Figure 6A and below. The photographs of the electrophoresis gels show the expected increasing occurrence of smaller protein fragments; 15 min after the start of enzymatic reaction (see Figure 7) no BSA is observed. Three strong bands indicating molecular weights around 30 kDa and some distinct bands around 20, 15, and 5 kDa can be seen. One of the three bands around 30 kDa can be correlated with subtilisin (ca. 27 kDa). During the following ten measurements the distribution of fragments broadens to give fragments with molecular weights of up to 30 kDa. After 3 h an almost even distribution

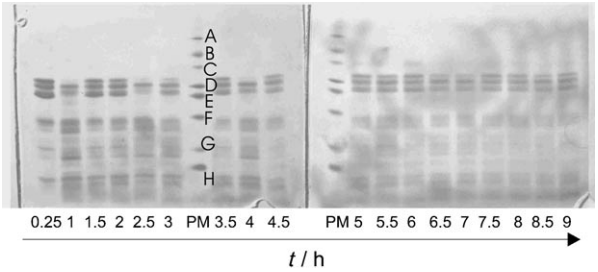

Figure 7. Gel electrophoresis of the reaction mixtures of 1 mM BSA digestion by subtilisin quenched at different times of reaction. Molecular weight of the protein marker (PM) proteins: A) 66, B) 45, C) 36, D) 29, E) 24, F) 20.1, G) 14.2, H) 6.5 kD.

is observed. During the following measurements a tendency to smaller fragments can be seen. After 5 h of reaction the gel shows a shift to smaller fragments well below 6 kDa. After 9 h the majority of protein fragments have a molecular weight smaller than 6 kDa as a result of hydrolytic cleavage of the protein. Due to their low weight they run out of the gel and cannot be detected anymore (gels not shown).

For practical use, for example, molecular screening applications, a correlation between molecular weight, reaction time, and fluorescence lifetime would be desired. In addition to Figure 6C and D, Figure 8 shows a correlation between molecular weight and reaction time. The intensities of the 30, 20, and

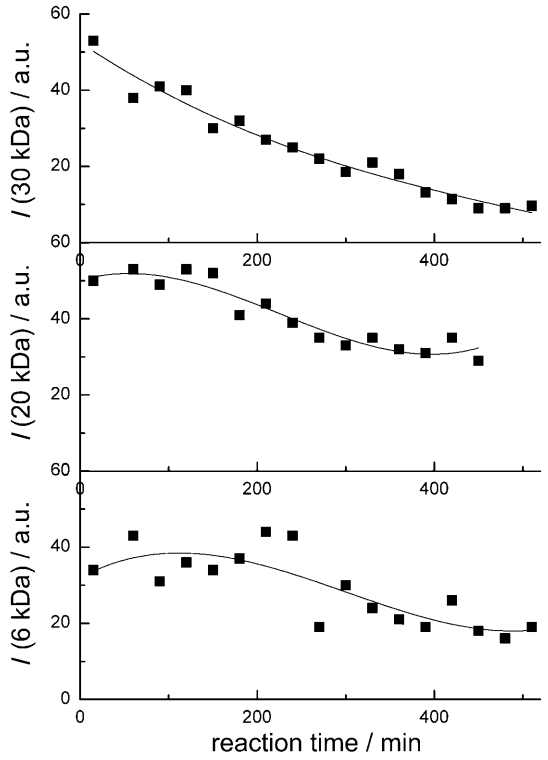

Figure 8. Intensity of the 3, 20, and 6 kDa bands of the electrophoresis gel as a function of the fluorescence lifetime of the reaction mixture. The solid line represents a cubic polynomial fit to the data and serves as a guide to the eye. The data represent measurements performed during the first 500 min of the reaction and correspond to the data shown in Figures 6A and C and 7.

6 kDa bands of the gel shown in Figure 7 were analyzed over the first 500 min of the reaction shown in Figure 6 A and C. This was achieved by using a horizontal intensity read out of the first two gel images monitoring the respective band by using simple photo software. The data were corrected with respect to the background between two adjacent bands. These intensity data of the three bands were plotted versus the reaction time to give Figure 8. A cubic polynomial least-squares fit to the data has been added for visional convenience.

Figure 8 gives a good impression of the origin of the decreasing fluorescence lifetime as well as a hint to how calibration of fluorescence lifetime to molecular weight distribution can be achieved. It can be seen that the intensity of the 30 kDa band decreases rapidly while that of 20 kDa band remains constant during the first measurements or even increases before decreasing much more slowly than that of the 30 kDa band. Although containing more noise, the 6 kDa band shows a similar yet even more pronounced behavior than the 20 kDa band.

As the evanescence of the 30 kDa band is much faster than those of the smaller fragments it can be seen that the distribution shifts to smaller fragments during the reaction, which then dominate the fluorescence lifetime. Furthermore, the fits to the data also imply the expected buildup in the concentration of the smaller fragments at the beginning of the reaction. Hence, a carefully performed gel electrophoresis with high resolution and subsequent intensity readout of each band over time can provide calibration data for correlation of the fluorescence lifetime of tryptophan with a molecular weight distribution.

3. Conclusions

We have presented a label-free method of observation for a proteolytic digestion using 2c2p excited intrinsic fluorescence of the involved protein. Two-color two-photon excitation using femtosecond laser pulses gives us a multiphoton technique with low photodamage, superior temporal resolution, intrinsic 3D resolution, and potentially high penetration depth. Additionally, 2c2p is a convenient technique for extending the effective wavelength range of the Ti:Sa femtosecond laser. In addition to the spectral windows for one photon (700–1000 nm) and conventional one-color two-photon techniques (350–500 nm), effective excitation from 233 to 333 nm can be achieved.

Changes in fluorescence lifetime of tryptophan depending on its environment have been reported earlier.[15,32] Additionally, numerous fluorescence assays have been published in which a shift in the fluorescence spectrum of tryptophan is observed on changing the surroundings of tryptophan.[33–35] These shifts also go hand in hand with a change in fluorescence lifetime.[36] Therefore, observing the digestion of tryptophan via fluorescence lifetime is just a first example demonstrating the power of a time-resolved 2c2p setup using femtosecond laser pulses from a Ti:Sa laser in monitoring protein reactions. This can be applied for a variety of different assays using intrinsic protein fluorescence.[39–41]

Moreover, the use of a multiphoton process for excitation makes this method ideal for microscopy applications where the use of high numerical aperture microscope objectives allows the laser power to be minimized. Due to the quadratic dependence of the excitation on the total excitation power, excitation is confined to a small focal volume. Hence, 2c2p microscopy combines intrinsic 3D resolution with dramatically reduced photodamage compared to conventional one-photon techniques. The wavelengths of 400 and 800 nm are considerably less harmful to biological samples than the corresponding 266 nm in the case of one-photon excitation. Using a large excess of 800 nm light allows minimization of the 400 nm power level. Hence, intrinsic UV fluorophores can be excited without UV irradiation. With respect to three-photon excitation the absolute power level can be kept comparatively low because two-photon absorption cross sections are much larger than three-photon ones.[37] With the help of femtosecond laser pulses this technique provides an ideal basis for time-resolved time-domain measurements with temporal resolution of only a few picoseconds. Since the main excitation power is provided by the 800 nm beam, a high penetration depth into biological samples can be expected, because near-IR is less scattered and absorbed in biological tissue than shorter wavelengths.[38] Due to the dependence of the excitation probability on the product of the intensities of the two excitation colors it is this lesser scattering and highly intense IR focus that determines the resolution in deep-tissue imaging. Application of this new 2c2p fluorescence microscopy technique will offer many new opportunities, for which the above-discussed label-free observation of protein digestion is just one example.

Experimental Section

The experimental setup is shown in Figure 1. Femtosecond laser pulses from a $NdYVO_4$-pumped Ti:Sa laser (mira 900 B, Coherent) are frequency-doubled by using a beta-barium borate (BBO) crystal. The second harmonic (400 nm) and the remaining fundamental (800 nm) are separated by a dichroic mirror. A linear stage is implemented to ensure exact temporal overlap of the laser pulses in the sample. The excitation power is adjusted by a lambda half-plate and thin-layer polarizer. After reunion of the two beams via a second dichroic mirror they are focused into the sample via an achromatic lens with a focal length of 5 cm. Spatial overlap can be adjusted by replacing the cuvette with a pinhole. Temporal overlap is adjusted by using a highly efficient 2c2p fluorescent dye, for example, *p*-terphenyl (PTP) in cyclohexane. 2c2p excitation is only present at spatial and temporal overlap of the excitation pulses of each color (Figure 2). The fluorescence is collected by a lens (L_2) with 25 mm focal length and a diameter of 30 mm. Optionally, after passing a set of filters (Semrock NF01-405U and Semrock NF01-808U, both at 10° angle, and a interference filter 340/80 nm), the fluorescence can be analyzed in terms of polarization by using a Rochon prism. Finally the fluorescence is projected onto a time-gated ICCD camera system (picostar, LaVision GmbH, Göttingen). The adjustable gate width (200 ps to 1 ns) in combination with a delay generator (Kentech Instruments Ltd.) allows a temporal resolution of about 10 ps.

BSA and subtilisin were purchased from Sigma-Aldrich and used without purification. A 1 mM and a 0.1 mM solution of BSA were

prepared with PBS buffer. Subtilisin was added to the protein solution to give a concentration of about 0.01 mM.

Gel electrophoresis was performed on polyacrylamide support matrices under conditions of discontinuous denaturation (125 V, 2 h) after quenching the enzymatic reaction by heat. As a ruler for molecular weight the protein marker M3913 from Sigma-Aldrich was used. The staining reagent was Coomassie Blue.

Acknowledgements

Financial support by the Deutsche Forschungsgemeinschaft (DFG) under grant no. Ge496/20 is gratefully acknowledged.

Keywords: amino acids · fluorescent probes · fluorescence spectroscopy · laser spectroscopy · time-resolved spectroscopy

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Received: September 5, 2008
Revised: November 11, 2008
Published online on January 20, 2009

