Quantum Control of Population Transfer in Green Fluorescent Protein by Using Chirped Femtosecond Pulses

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Abstract: We demonstrate that the methods of quantum control can be applied successfully to very large molecules in room temperature liquid solution. Chirped femtosecond pulses are used to excite a green fluorescent protein mutant in both buffered aqueous solution and solid acrylamide gel. At high energy densities, the fluorescence shows a strong chirp dependence, with positively chirped pulses transferring almost 50% more population to the excited state than negatively chirped pulses. By measuring the photobleaching rate in the gel as a function of pulse chirp, we find that the data are consistent with the bleaching of the protein being due to a thermal mechanism rather than to an excited-state photoreaction.

Introduction

Pulsed nuclear magnetic resonance (NMR) spectroscopy is arguably the most powerful spectroscopic tool of the present day chemist. Much of the power of this technique results from the ability of the experimenter to exquisitely control the shapes of the radio frequency pulses used to excite and probe the sample. Long ago it was realized that in principle similar pulse shaping techniques could benefit optical spectroscopy, and pioneering experiments showed both the promise and the limitations of the optical-NMR analogy.[1,2] While the use of shaped optical pulses as routine spectroscopic probes is still in the future, recent advances in ultrafast laser technology and optical pulse shaping techniques have led to renewed interest in the use of such pulses for the manipulation of molecules. This field, known as "quantum control", began as a predominantly theoretical exercise, but is rapidly becoming an experimental reality.[3]

The simplest way to shape an optical pulse is to modify its coherent phase structure or "chirp" by using optical elements such as gratings, prisms, or bulk material. A short pulse has many frequency components, and putting chirp on the pulse delays some of those components with respect to others in the time domain. A positively chirped pulse has low frequencies leading and high frequencies trailing, while a negatively chirped pulse has the opposite frequency ordering. For a zero chirp or transform-limited pulse, all the frequencies arrive at the same time. Note that we can modify the phase structure of the laser pulse, and thus its temporal properties, without changing observables such as pulse energy or the power spectrum. Such chirped or shaped light pulses have been used to optimize population transfer,[4−6] control wave packet dynamics,[7−9] and obtain information about vibrational dynamics in four-wave-mixing experiments.[10,11] The usefulness of chirped pulses has been demonstrated not only for atoms and small molecules in the gas phase, but also for large organic molecules in room-temperature liquids.[12] A natural question arises as to whether there is a limit to the size of the molecular system that may be "controlled" before the many degrees of freedom wash out effects due to the coherent phase structure of the light field.

In this paper we show that molecules at least as large as a protein can be manipulated by using chirped pulses. Intense chirped femtosecond pulses are used to control the fluorescence output of a particular mutant of the green fluorescent protein (GFP). This mutant, specifically S65G/S72A/T203Y, which we shall refer to as the Yellow Fluorescent Protein (YFP) since its emission is actually yellow, is a 27 kdalton protein that is of interest for its applications in protein tagging and biological imaging.[13] By varying the chirp of the exciting pulse, we demonstrate that the fluorescence signal can by varied by almost 50% at high photon flux. By immobilizing the protein in a porous gel and measuring the photobleaching dynamics as a function of chirp, we find that the photobleaching dynamics are consistent with a thermal process on the ground state, rather

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(1) Warren, W. S.; Zewail, A. H. *J. Chem. Phys.* **1983**, *78*, 2279−2297.
(2) Warren, W. S. *Science* **1988**, *272*, 878−884.
(3) Gordon, R. J.; Rice, S. A. *Annu. Rev. Phys. Chem.* **1997**, *48*, 601−641.
(4) Melinger, J. S.; Gandhi, S. R.; Hariharan, A.; Goswami, D.; Warren, W. S. *J. Chem. Phys.* **1994**, *101*, 6439−6454.
(5) Cerullo, G.; Bardeen, C. J.; Wang, Q.; Shank, C. V. *Chem. Phys. Lett.* **1996**, *262*, 362−368.
(6) Assion A.; Baumert, T.; Helbing, J.; Seyfried, V.; Gerber, G. *Chem. Phys. Lett.* **1996**, *259*, 488−494.
(7) Kohler, B.; Yakovlev, V. V.; Che, J.; Krause, J. L.; Messina, M.; Wilson, K. R.; Schwentner, N.; Whitnell, R. M.; Yan, Y. J. *Phys. Rev. Lett.* **1995**, *74*, 3360−3363.
(8) Bardeen, C. J.; Che, J.; Wilson, K. R.; Yakovlev, V. V.; Apkarian, V. A.; Martens, C. C.; Zadoyan, R.; Kohler, B.; Messina, M. *J. Chem. Phys.* **1997**, *106*, 8486-8503.
(9) Papanikolas, J. M.; Williams, R. M.; Leone, S. R. *J. Chem. Phys.* **1997**, *107*, 830−830.
(10) Duppen, K.; de Haan, F.; Nibbering, E. T. J.; Wiersma, D. A. *Phys. Rev. A* **1993**, *47*, 5120−5137.
(11) Bardeen, C. J.; Wang, Q.; Shank, C. V. *Phys. Rev. Lett.* **1995**, *75*, 3410−3413.
(12) Bardeen, C. J.; Yakovlev, V. V.; Wilson, K. R.; Carpenter, S. D.; Weber, P. M.; Warren W. S. *Chem. Phys. Lett.* **1997**, *280*, 151−158.
(13) Phillips, G. N. *Curr. Opinion Struct. Biol.* **1997**, *7*, 821−827.

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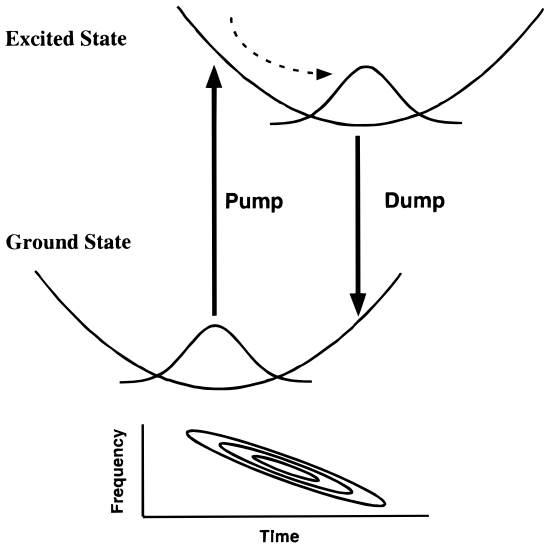

Figure 1. Schematic of the pump−dump sequence of photon interactions that leads to the dependence of the excited state population on the pulse chirp. The upper part of the figure shows the two electronic states of the molecule, with nuclear degrees of freedom represented as harmonic potentials. As the initially excited population slides down the potential energy surface, a negatively chirped pulse, represented in frequency and time in the lower part of the figure, can dump it back to the ground state. A positively chirped pulse (not shown) discriminates against this process, and population is trapped on the excited state.

than an excited-state reaction. Thus we demonstrate that the techniques of quantum control are applicable to large biological systems, e.g. the YFP, and that we can use these techniques to learn about the behavior of such systems.

A brief explanation of the effect of chirped pulses in exciting fluorescence from one-photon transitions is as follows. The full structure of the pulse can be viewed in a two-dimensional representation, such as the Wigner plot shown in Figure 1, where one axis is optical frequency and the other is time. For instance, a positively chirped pulse has a positive slope in such a plot, with frequency increasing as a function of time. When such a pulse interacts with a molecular system, it excites population onto the excited state. This initial population is generally nonstationary, since it is in a nonequilibrium position on the excited state potential energy surface, and rapidly relaxes to lower energies due to solvent reorganization and internal relaxation. If a lower energy photon then interacts with this relaxing or fully relaxed population, it can stimulate emission and put the molecule back on the ground state. Figure 1 shows a schematic of this "pump−dump" process for a negatively chirped pulse, where low frequencies trail high and such a process is favored. A positively chirped pulse, with the opposite frequency ordering, discriminates against this process and maximizes the amount of population left on the excited state. It is this dependence of the excited-state population on chirp, as measured by the total fluorescence signal, that we observe in YFP. This decrease in spontaneous emission with negative chirp is accompanied by an increase in stimulated emission due to the "dump" part of the pulse.[5] It should be emphasized that this analysis assumes that only two electronic states, the ground and first excited state, are spectroscopically coupled. In a large organic molecule, sequential absorption to a higher lying excited state may be possible. But while it is difficult to rule out such excited-state absorptions in the absence of detailed spectroscopic data, their predicted effect on the chirp dependence of the fluorescence is different from that of sequential absorption and emission. Even if the excited-state absorption was enhanced by

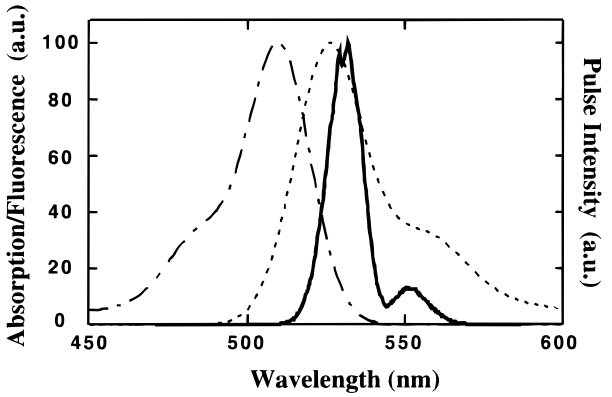

Figure 2. Absorption (dot−dashed line) and emission (dashed line) spectra of the T203Y mutant of the green fluorescent protein (YFP), along with the pulse spectrum (solid line).

the appropriate pulse chirp, according to Kasha's rule the population on the higher excited state would quickly relax to the lowest excited state and contribute to the fluorescence signal as if nothing had happened. In other words, chirp-dependent excited-state absorption would be expected to have little effect on the total fluorescence signal, unlike chirp-dependent stimulated emission, which depletes the fluorescent state permanently.

Experimental Section

The laser system used in these experiments is described elsewhere.[8] Briefly, the output of a regeneratively amplified kilohertz Ti:Sapphire laser system is used to pump an optical parametric amplifier (OPA) operating in the near-infrared. By tuning a birefringent filter in the amplifier cavity, we shift the output pulses to shorter wavelengths, around 780 nm, which in turn allows the OPA to operate at shorter wavelengths. The output of the OPA is frequency doubled in a BBO crystal and the resulting second harmonic is tunable between 530 and 650 nm.[14] A typical pulse spectrum at 530 nm, along with the excitation and emission spectrum of YFP, is shown in Figure 2. The output pulse passes through approximately 20 cm of BK7 optical glass, and the resulting positive chirp is then compensated by double passing the beam in a pair of 300 line/mm gratings. By changing the separation of the gratings, we can adjust the linear chirp over a wide range.[15] The pulses are measured by using a noncollinear polarization frequency resolved optical gating (FROG) technique.[16,17] The two beams in an autocorrelator are focused into a 1 mm thick piece of fused silica. The polarization rotation of the probe beam due to the nonlinear Kerr effect induced by the pump beam is detected and spectrally resolved in a monochromator coupled to a CCD detector. The resulting data sets are analyzed by using a standard FROG retrieval algorithm. When the grating separation is set so that the linear dispersion of the BK7 is exactly canceled by the gratings, the retrieved intensity fwhm is 50 fs, limited by cubic phase distortion. Thus the shortest pulse has no linear chirp, but does have residual higher order chirp terms and is not strictly transform-limited. As the separation of the gratings is changed, the linear term dominates over the higher order terms and the pulse lengthens symmetrically, as expected. For instance, at $\phi'' = \pm 5000$ fs^2, the pulse width is approximately 300 fs, as determined by FROG measurements.

Concentrated YFP is obtained in a pH 7.5 buffered solution and diluted to a concentration of 1.5 μg/μL with use of pH 7.5 HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]) buffered solution. A small amount of this solution is placed in a 200 μm path length, rectangular glass capillary tube and this is the sample for the fluorescence experiments. For the photobleaching experiments, the YFP

(14) Wilson, K. R.; Yakovlev, V. V. *J. Opt. Soc. Am. B* **1997**, *14*, 444−448.
(15) Treacy, E. B. *IEEEJ. Quantum Electron.* **1969**, *5*, 454−458.
(16) Trebino, R.; Kane, D. J. *J. Opt. Soc. A* **1993**, *10*, 1101−1111.
(17) Kohler, B.; Yakovlev, V. V.; Wilson, K. R.; Squier, J.; DeLong, K. W.; Trebino, R. *Opt. Lett.* **1995**, *20*, 483−485.

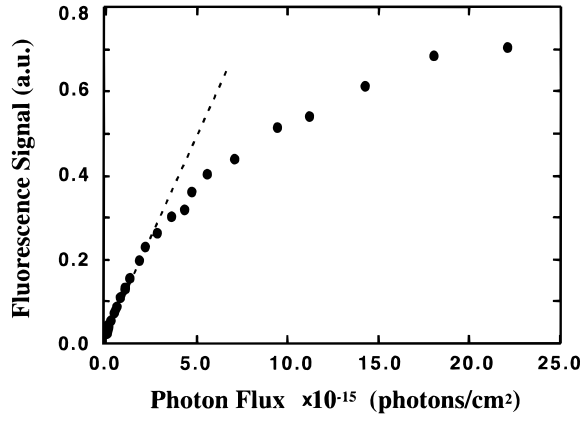

Figure 3. Dependence of the fluorescence of the YFP on the pulse energy, showing the onset of saturation, i.e., a deviation from the linear behavior indicated by the dashed line, at a pulse energy corresponding to a photon flux of approximately 5×10^{15} photons/cm^2.

is immobilized in an polyacrylamide gel matrix.[18] Typically, 9 μL of the dilute YFP solution was mixed with 10 μL of 40% (19:1) acrylamide:bisacrylamide solution (Accugel, National Diagnostics) and 1 μL of 1% ammonium persulfate solution and the mixture was placed in the capillary tube, which is then dipped into a 4% solution of TEMED (*N*,*N*,*N*′,*N*′-tetramethylethylenediamine) and distilled water. The small amount of TEMED soaked up by the capillary catalyzes the gel polymerization and leads to a solid sample containing YFP molecules whose spectral characteristics are similar to those of the protein in aqueous solution.[19]

Both the fluorescence and photobleaching experiments are done by focusing the excitation beam, with a diameter of 4 mm and a maximum energy of 25 nJ, into the sample by using a 10 cm focal length lens. Assuming diffraction-limited focusing, the maximum peak intensity in the sample is thus 1.7×10^{11} W/cm^2. The maximum energy density per pulse is approximately 0.9 mJ/cm^2, corresponding to a photon density of 2.2×10^{16} photons/cm^2. The fluorescence perpendicular to the excitation beam is collected with a 20× objective lens and imaged onto a photomultiplier tube. No attempt is made to spatially resolve the emission. To prevent laser scatter from affecting these measurements, a cutoff filter at 590 nm is used in combination with a 589 nm interference filter (3 nm bandwidth) so that only a narrow part of the YFP's long wavelength fluorescence is detected by the PMT. Measurements of the long wavelength portion of the fluorescence spectrum with a CCD show no change in the spectral shape when the chirp of the exciting pulse is changed, implying that we are observing only fully relaxed fluorescence. Both the fluorescence signal and the excitation power are monitored with use of lock-in detection.

Results and Discussion

The dependence of the fluorescence output on pulse energy for pulses of zero linear chirp is shown in Figure 3. The departure from a linear dependence at a pulse energy of about 5 nJ, corresponding to a photon flux of 4×10^{15} photons/cm^2, signals the onset of saturation. Note that the peak intensity of the femtosecond pulse corresponding to this photon flux (3.1×10^{10} W/cm^2) is well above that at which saturation would be expected with continuous excitation, but since the sample only experiences this intensity for a short time, we do not invert the entire population. The saturation observed in these experiments is due instead to the pump−dump processes discussed in the Introduction, which in turn depend on the details of the pulse spectrum and chirp.

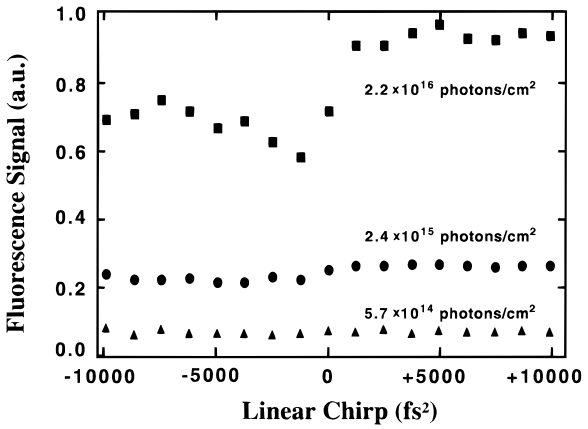

Figure 4. Chirp dependence of the measured fluorescence of the YFP at several different pulse energies (photon fluxes): 0.7 nJ (5.7×10^{14} photons/cm^2), triangles; 2.7 nJ (2.4×10^{15} photons/cm^2), circles; and 25 nJ (2.2×10^{16} photons/cm^2), squares.

Figure 4 shows the change in the fluorescence signal of the YFP in solution as a function of pulse chirp at various pulse energies, from the linear to the saturated regime. The first important point is how the chirp behavior depends on pulse energy. For the lowest energy pulses, we see that there is little or no dependence of the fluorescence on chirp. In the low-energy, linear-response regime, with a photon flux of 6×10^{14} photons/cm^2, the pump−dump sequence of photon interactions described in the Introduction has a low probability and the relative ordering of the frequency components does not matter. In this regime, a YFP molecule on average only absorbs a single photon, and rarely interacts with a second. At higher pulse energies, the pump−dump sequence becomes more likely, and the asymmetry with chirp begins to appear. At the highest pulse energies, where we are well into the saturated regime, there is a dramatic change in the fluorescence output of the YFP as we change the chirp, with positively chirped pulses resulting in 50% more fluorescence than negatively chirped pulses of the same energy. Note that this chirp dependence is consistent with a saturation process dominated by absorption and emission, rather than by absorption and excited-state absorption. These data show how pulse coherence effects can become important at higher intensities where multiple photon interactions become more likely. As low intensities, the phase of the pulse is unimportant, as predicted by the Brumer−Shapiro Emperor's New Clothes Theorem.[20] This theorem simply states that interference effects (which are due to pulse phase terms) require at least two photons interacting with the sample and has been used to show that quantum control of population distributions cannot be obtained in the low-power, one-photon limit, no matter what the shape of the pulse.

One point worthy of discussion is the dip in the high-energy fluorescence data at slight negative chirps. This dip is reproducible and recent theoretical work[21] suggests that it results from excited-state dynamics. Consider Figure 1: if the excited state wave packet dynamics are much faster than any pulse dynamics, then the molecule is essentially a two-state system and whether a pump−dump process is possible depends only on the sign of the chirp. In other words, the fluorescence dependence on chirp should look like a step function centered at zero chirp. If, on the other hand, the excited-state relaxation is not instantaneous, then there will be an optimum negative chirp that can follow the motion of this nonequilibrium population as it relaxes. Such

(18) Dickson, R. M.; Norris, D. J.; Tzeng, Y. L.; Moerner, W. E. *Science* **1996**, *274*, 966−969.
(19) Dickson, R. M.; Cubitt, A. B.; Tsien, R. Y.; Moerner, W. E. *Nature* **1997**, *388*, 355−358.
(20) Brumer, P.; Shapiro, M. *Chem. Phys.* **1989**, *139*, 221−228.
(21) (a) Cao, J.; Bardeen, C. J.; Wilson, K. R. *J. Chem. Phys*. Submitted for publication. (b) Fainberg, B. D. *J. Chem. Phys.* **1998**, *109*, 4523−4532.

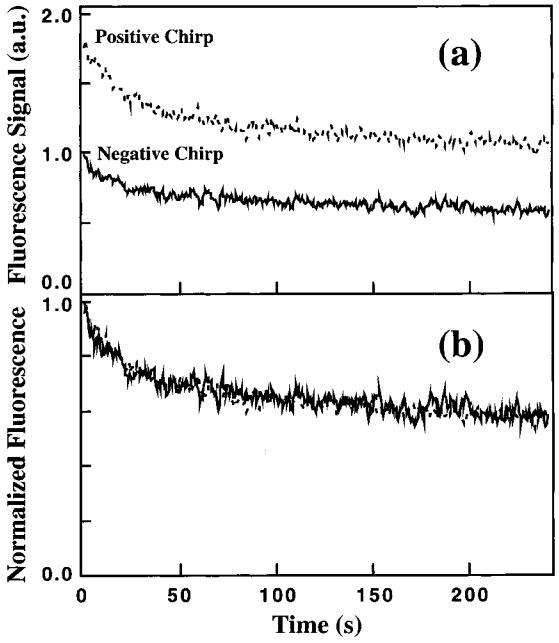

Figure 5. (a) Observed decays of fluorescence signal from YFP in a gel matrix irradiated by positively (dashed line) and negatively (solid line) chirped pulses. (b) Same as in part a, but with the decays normalized to the average fluorescence signal.

a chirp will result in a minimum of fluorescence, which is what we observe in Figure 4 at $\phi'' = -1200$ fs^2. Absolute confirmation that this is the optimal "dump" chirp would be provided by a measurement of the stimulated emission collinear with the excitation pulse.[5] Although there is an optimal chirp for minimizing fluorescence, the maximum of the fluorescence is basically constant for any positive chirp greater than +1000 fs^2. This robust quality of positively chirped pulses to maximize population inversion has previously been observed both experimentally and theoretically.[5,12,22,23] Note that both of these chirps correspond to pulses that are still relatively short, with durations on the order of 100 fs or less.

The fact that we can change the excited state population of the YFP by modifying the pulse chirp, without changing the energy or spectral content, suggests that we might also be able to modify a photochemical reaction rate in this way. If there is a photobleaching reaction that occurs on the excited state of the molecule, then a pulse that leaves more molecules on the excited state will accelerate the photobleaching process. Thus the rate of photoreaction for a positively chirped pulse should be greater than that for a negatively chirped pulse, since the latter pulse leaves less population on the excited state. We test this idea on YFP by immobilizing it in a gel matrix and monitoring the rate of decay of the fluorescence as it is irradiated by a pulse with a given chirp. Using a pulse centered at 545 nm leads to a difference in fluorescence signal of a factor of 2 when positive as opposed to negative chirp is used. Since a positively chirped pulse leaves a factor of 2 times more population on the excited state than does a negatively chirped pulse, the total number of molecules should decay more quickly under irradiation by a positively chirped pulse. The decay of the fluorescence takes place on a time scale of seconds and the two curves, corresponding to linear chirps of ± 4800 fs^2, are shown in Figure 5a. The curves have been normalized for purposes of comparison in Figure 5b. To within the experimental accuracy, they are identical.

The fact that the photobleaching rates are identical for positive and negative chirps strongly implies that excited-state photobleaching plays a minor role. This conclusion is supported by the results of other workers who found no indication of excited-state reactions with O_2 or triplet quenchers for a different GFP mutant.[24] The isolation of the GFP chromophore in its protein matrix may preclude it from encountering environmental reactants during its residency on the excited state, and this same protein matrix may also provide a mechanism for ground state photobleaching. While positively and negatively chirped pulses leave different amounts of population on the excited state, as measured by the fluorescence, we expect that both pulses deposit similar amounts of heat in the sample. This is because whether a molecule falls back to the ground state via spontaneous or stimulated emission, in either case it has undergone a Stokes shift that has left a significant amount of heat in the sample. Thus we would not expect to see a chirp effect if the photobleaching is due to a thermally activated reaction on the ground state, as suggested by Moerner and co-workers.[19] This analysis assumes that most of the relaxation (and concomitant heat deposition) occurs within the chirped pulse width. For small negative chirps, Figure 4 suggests that it may be possible to dump the excited-state population before it has completely relaxed. In this case it might be possible to observe an effect on the photobleaching rate, since less heat would be left in the sample.

The exact mechanism of GFP photobleaching is not well-understood, and in general may involve a number of ground- and excited-state reactions.[25] In any event, the lack of any chirp dependence in the observed photobleaching rate of the YFP in the gel suggests that the mechanism of photodestruction, for excitation at 545 nm at least, is not due to excited-state photochemistry. From Figure 2 we see that 545 nm is in the low-energy wing of the absorption spectrum, and it is possible that excitation at shorter wavelengths, which results in excited-state populations with more excess energy, might result in some excited-state chemistry. Tuning the OPA as far to the blue as possible (530 nm) yielded no discernible chirp effect, however.

Conclusions

Only a few years ago, quantum control was largely a theoretical concept, applied only to small, isolated molecular systems in the gas phase.[3] The data presented here clearly show that quantum control techniques may be applied to molecules at least as large as proteins in complicated environments such as room temperature liquids. By changing the chirp of the excitation pulse, the electronic state population transfer in a large protein can be controlled in a manner similar to that which has been demonstrated in smaller molecules. Application of chirped pulses to the photobleaching of YFP has also provided clues to the mechanism of photobleaching, suggesting that it is thermal in origin and is an unavoidable consequence of laser heating. This work demonstrates that the techniques of quantum control may be used not only to control molecules, but also to obtain useful information about their dynamics. Recent advances in ultrafast pulse shaping technology[26−29] should make it possible

(22) Cao, J.; Bardeen, C. J.; Wilson, K. R. *Phys. Rev. Lett.* **1998**, *80*, 1406−1409.
(23) Ruhman, S.; Kosloff, R. *J. Opt. Soc. B* **1990**, *7*, 1748−1752.
(24) Swaminathan, R.; Hoang, C. P.; Verkman, A. S. *Biophys. J.* **1997**, *72*, 1900−1907.
(25) Brejc, K.; Sixma, T. K.; Kitts, P. A.; Kain, S. R.; Tsien, R. Y.; Ormo, M.; Remington, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 2306−2311.

to extend the capabilities of optical spectroscopy and quantum control even further.

(26) Weiner, A. M.; Heritage, J. P.; Kirschner, E. M. *J. Opt. Soc. B* **1988**, *5*, 1563−1572.
(27) Wefers, M. M.; Nelson, K. A. *Opt. Lett.* **1993**, *18*, 2032−2034.
(28) Dugan, M. A.; Tull, J. X.; Warren, W. S. *J. Opt. Soc. B* **1997**, *14*, 2348−2358.
(29) Bardeen, C. J.; Yakovlev, V. V.; Wilson, K. R.; Carpenter, S. D.; Weber, P. M.; Warren, W. S. *Chem. Phys. Lett.* **1997**, *280*, 151−158.

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