Picosecond Time-Resolved Fluorescence from Blue-Emitting Chromophore Variants Y66F and Y66H of the Green Fluorescent Protein

Andreas D. Kummer,[a]+ Jens Wiehler,[b]+ Tanja A. Schüttrigkeit,[a] Bryan W. Berger,[a, c] Boris Steipe,[b, d] and Maria E. Michel-Beyerle*[a]

The origin of the low steady-state fluorescence quantum yield of some blue-emitting variants of the green fluorescent protein (GFP) is investigated in single-site mutants in which the tyrosine residue at position 66 has been replaced by phenylalanine or by histidine. Time-resolved fluorescence measurements reveal excited-state lifetimes of 74 ps (Y66F) and 0.9 ns (Y66H) at room temperature that increase to values close to the radiative limit as the temperature is lowered. These short lifetimes are explained by temperature-dependent internal conversion. The pronounced difference between the room-temperature lifetimes of the two mutants suggests that hydrogen bonding of the distal aromatic ring plays a more important role than tight packing in the fixation of the chromophore.

KEYWORDS:

fluorescence · internal conversion · kinetics · proteins · time-resolved spectroscopy

Introduction

The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* is a unique and widely applied tool in molecular biology and biotechnology as a result of its strong intrinsic emission.[1] The GFP chromophore is formed from the tripeptide motif Ser65-Tyr66-Gly67 by an autocatalytic reaction in the presence of molecular oxygen[2, 3] and is tightly packed and protected from the bulk solvent in a can-shaped tertiary structure.[4] This complex but precisely defined protein environment is responsible for the rich photodynamic behavior observed in native GFP and its genetically engineered mutants.

The main absorption bands of wild-type GFP at 397 nm and 477 nm have been attributed to a ground state equilibrium between two species of the chromophore that differ in the protonation state of the Tyr66 residue.[2, 3] Excitation of the phenolic form at around 400 nm triggers excited-state deprotonation on the picosecond time scale, which leads to a species with a long-lived (3.3 ns) green emission almost identical to that obtained after direct excitation of the phenolate form at around 480 nm.[5–7] When the chemical nature of the chromophore does not allow deprotonation, for example, when the Tyr66 residue is replaced by a phenylalanine or histidine residue, blue fluorescence is observed at around 450 nm.[2, 3, 6, 8] These blue fluorescent proteins (BFPs) were among the first GFP mutants with spectral characteristics sufficiently altered to permit double labeling in protein expression[9] and fluorescence resonance energy transfer (FRET) experiments[10, 11] solely based on this class of proteins. However, the usefulness of BFPs is limited by their low fluorescence quantum yield (for example, 0.24 and 0.013 for the mutants Y66H and Ala1b/F64L/Y66F/Q80R/I167T/K238N, respectively)[12] and by rapid photobleaching, especially in the case of the single-site mutants.[2, 3, 8] Based on crystallographic structural information for variants that contain the amino acid mutation Tyr66 to phenylalanine (Y66F) or to histidine (Y66H) in a multiply mutated environment,[12–14] it was proposed that increased flexibility of the active site and hence a loss of quantum yield may cause this reduced fluorescence intensity,[12, 15] rather than inefficient protein folding or chromophore formation as suggested earlier for the Y66W mutant.[3]

In this paper we investigate the origin of the low fluorescence quantum yield in picosecond time-resolved fluorescence experiments on the single-site mutants Y66F and Y66H. The results

[a] *Prof. Dr. M. E. Michel-Beyerle, Dr. A. D. Kummer,+ Dipl.-Chem. T. A. Schüttrigkeit, B. W. Berger Institut für Physikalische und Theoretische Chemie Technische Universität München Lichtenbergstrasse 4, 85748 Garching (Germany) Fax: (+49) 89-289-13026 E-mail: michel-beyerle@ch.tum.de*

[b] *Dr. J. Wiehler,+ Prof. Dr. B. Steipe Genzentrum der Ludwig-Maximilians-Universität München Feodor-Lynen-Strasse 25, 81377 München (Germany) Fax: (+49) 89-2180-6999*

[c] *B. W. Berger Current address: Center for Molecular and Engineering Thermodynamics Department of Chemical Engineering, University of Delaware Newark, DE 19716 (USA) Fax: (+1) 302-831-4466*

[d] *Prof. Dr. B. Steipe Current address: Department of Biochemistry Program for Proteomics and Bioinformatics University of Toronto 1, King's College Circle, Toronto, Ontario, M5S 1A8 (Canada)*

[+] *These authors contributed equally to this work.*

indicate that the predominant channel for loss of excitation energy depends in a sensitive way on hydrogen bond interactions between the chromophore and its immediate protein environment. The free volume of the chromophore has to be taken into account as well, but plays a secondary role.

Results and Discussion

The mutants Y66F and Y66H: Steady-state absorption and fluorescence

Steady-state absorption and fluorescence emission spectra of the single-site mutants Y66F and Y66H at 150 K are depicted in Figure 1. Both mutants exhibit two absorption bands in the UV/Visible region of the spectrum. The higher energy band at 277 nm arises from the presence of aromatic residues in the protein barrel[16] and may include minor contributions from higher electronic transitions of the chromophore as concluded from comparison with spectra of model compounds.[17, 18] The low energy band, with its maximum at 355 nm for Y66F and 379 nm for Y66H, pertains to the S0 → S1 transition of the chromophore. Shape and position of the corresponding fluorescence emission spectra are almost invariant with respect to excitation wavelength in the range 300 – 400 nm, except for a small red shift of less than 4 nm when excitation is in the red wing of the absorption band. The emission spectra are more structured than the absorption spectra and the emission maximum is reached at 427 nm for Y66F and 442 nm for Y66 H. The excitation spectra of this blue fluorescence closely follow the absorption band of the chromophore (not shown). In general, the spectra become less structured with increasing temperature but no essential change in peak positions is observed apart from a slight red shift of a few nanometers. At room temperature the spectra are invariant with the addition of cryoprotector (50 % glycerol). Even a superficial comparison indicates that the fluorescence intensity is dependent on temperature.

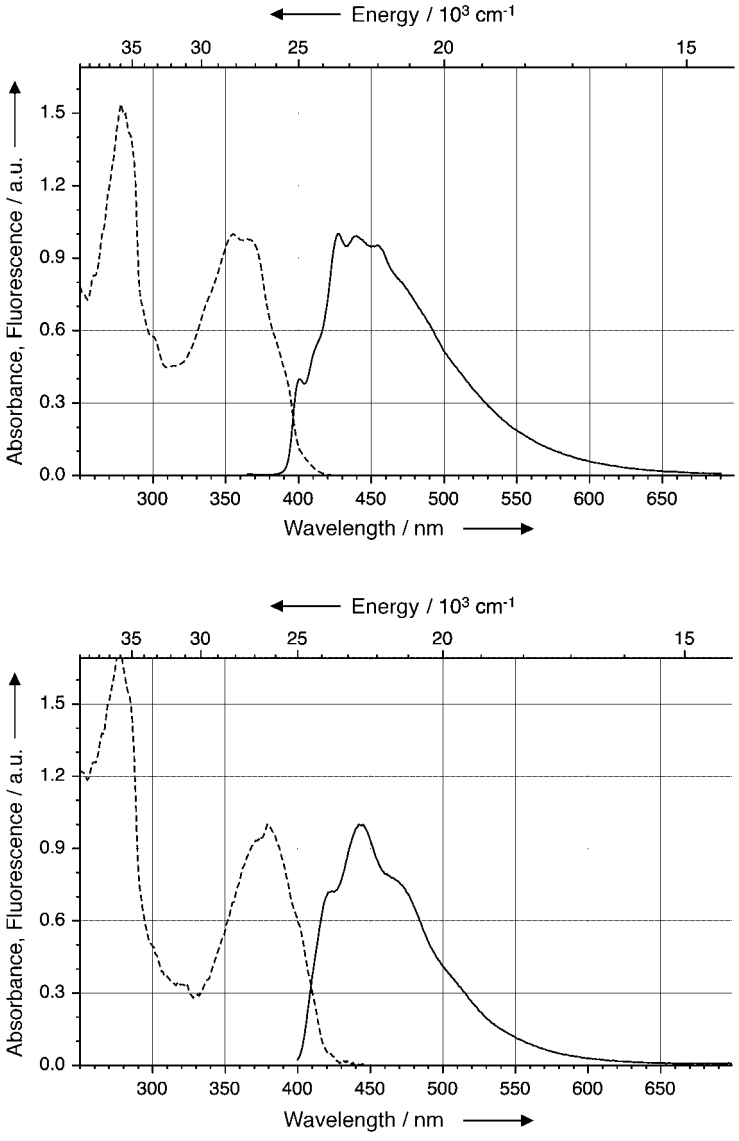


Figure 1. *Steady-state absorption (dashed line) and fluorescence emission spectra (solid line) of the single-site mutants Y66F (top, excitation wavelength $\lambda_{exc} = 355$ nm) and Y66H (bottom, $\lambda_{exc} = 379$ nm) at 150 K.*

The mutant Y66F: Time-resolved fluorescence

This mutant fluoresces only weakly at 295 K whereas a strong blue emission is obtained at 80 K. Picosecond time-resolved fluorescence measurements (Figure 2 and Table 1) reveal a fluorescence signal which appears instantaneously within the time resolution of our experiments (see the Materials and Methods Section) and decays nonexponentially at all the investigated temperatures ($\geq$ 80 K) with time constants that span the pico- to nanosecond range. Even at 80 K, where comparatively longer time components predominate, in most cases a time constant of less than 50 ps is necessary to achieve a good fit. The averaged lifetime,[19] $\langle t \rangle = \Sigma A_i t_i$, of the S1 state is increased by a factor of more than 50 by the temperature change, that is, from about 70 ps at 295 K to around 3.6 ns at 80 K. In addition, the fluorescence kinetics seem to be slightly faster when detected in either wing of the emission band than when observed at the spectral maximum (data not shown).

According to classical theory, the radiative lifetime of the GFP chromophore has been estimated from the extinction coefficients of absorption to be in the range of a few nanoseconds. In wild-type GFP, the fast decay of the blue fluorescence at 450 nm ($\langle t \rangle = 29$ ps) has been shown to be caused mainly by excited-state proton transfer (ESPT) to a nearby proton acceptor.[5, 6] Internal conversion plays a secondary role; the average time constant of internal conversion has beeen estimated to be of the order of 0.2 ns.[20] In the mutant Y66F the deprotonating hydroxy group at position 66 is replaced. Therefore, in the absence of ESPT, we attribute the short fluorescence lifetime of 74 ps at room temperature to enhanced internal conversion. Two structural features support this interpretation. First, the missing hydroxy group leaves a cavity adjacent to the chromophore.[12] As suspected previously,[12] the enlarged free volume

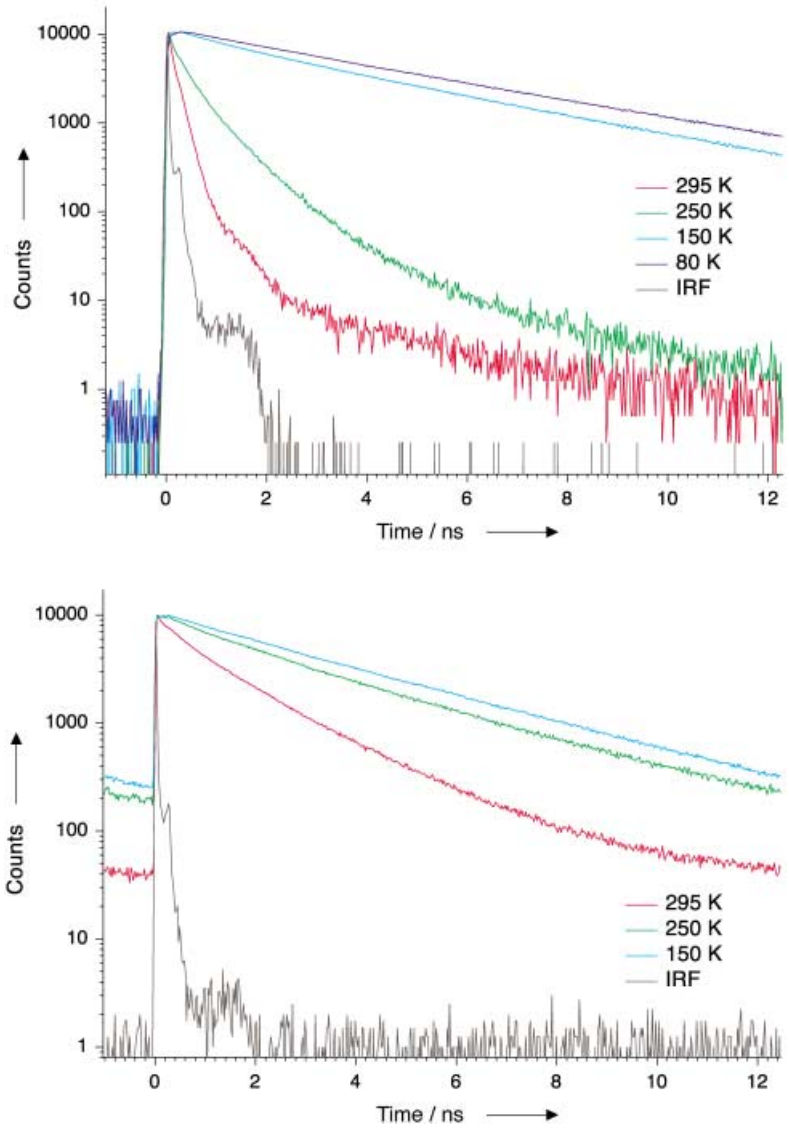


Figure 2. *Fluorescence decay of the excited chromophore in the single-site mutants Y66F (top, $\lambda_{exc} = 359$ nm, detection wavelength $\lambda_{det} = 430$ nm) and Y66H (bottom, $\lambda_{exc} = 370$ nm, $\lambda_{det} = 450$ nm) at selected temperatures. The instrument response function (IRF) had a full width at half-maximum (fwhm) of 40 ps and 30 ps, respectively.*

Table 1. *Fit results for selected fluorescence measurements of the single-site GFP mutants Y66F and Y66H.*[a]

Temperature [K]	t_1 [ps]	A_1 [%]	t_2 [ps]	A_2 [%]	t_3 [ps]	A_3 [%]	t_4 [ns]	A_4 [%]	$\langle t \rangle$ [ns]
Y66F									
295	14	47	86	35	200	17	1.29	0.2	74[c]
250	43	43	214	31	636	25	1.79	1	272[c]
150	14	47	288	6	1.49[b]	13	4.17	33	1.60
80	15	12			1.48[b]	12	4.57	75	3.63
Y66H									
295			78	31	666	29	1.82	40	945[c]
250					386	20	2.81	80	2.33
150					804	12	3.50	88	3.17

[a] $\lambda_{exc} = 359$ nm, $\lambda_{det} = 430$ nm for the Y66F mutant and $\lambda_{exc} = 370$ nm, $\lambda_{det} = 450$ nm for the Y66H mutant. Samples were in 50% glycerol. Amplitudes are normalized to 100%. The averaged lifetime is calculated as $\langle t \rangle = \Sigma A_i t_i$. [b] Values in ns. [c] Values in ps.

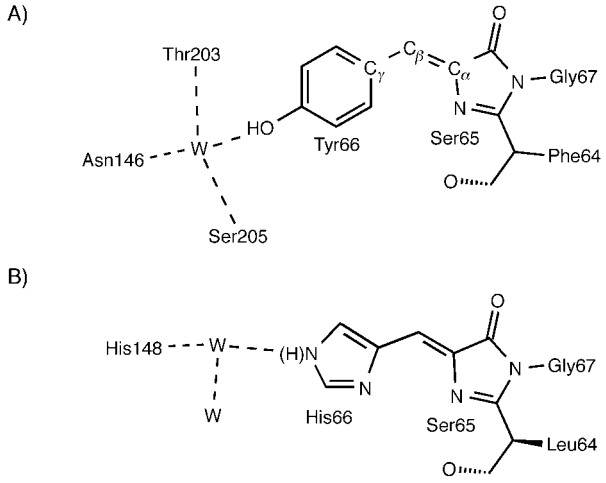
may indeed favor rotational motion within the chromophore skeleton. Second, in wild-type GFP the sp^2-hybridized phenolic oxygen atom of the Tyr66 residue forms hydrogen bonds with the protein matrix through a water molecule (Scheme 1 A), which may restrict the rotational freedom of the distal aromatic ring. This hydrogen bonding is absent in the Phe66 variant.

The fluorescence decay shows dispersive kinetics that are strongly temperature dependent over the whole temperature range investigated. This feature qualitatively reproduces the strong temperature dependence of the nonexponential fluorescence decay of a model compound of the chromophore dissolved in glycerol. This decay has been demonstrated to depend predominantly on the macroscopic viscosity of the solution rather than on thermal activation of the rotational motion within the chromophore.[21] The nonexponential decay pattern as well as its spectral dependence reflect structural disorder. The observation that the shortest decay time constant is maintained down to the lowest temperature (80 K) with relatively modest attenuation of its amplitude is characteristic of a specific protein configuration that allows for the most efficient rotational motion between the two aromatic moieties of the chromophore.

The mutant Y66H

In the mutant Y66H, the dependence of fluorescence lifetime on temperature is less pronounced (Figure 2 and Table 1) than for the Y66F mutant, although the general dispersive kinetics features are similar. At room temperature an averaged lifetime $\langle t \rangle$ of 0.9 ns was measured for the S1 state of the Y66H mutant. Cooling to 150 K causes a deceleration by a factor of approximately three.

Similar mutants that carry a His66 residue have been subjected to crystallographic studies by different research groups.[12–14] Since our experiments were performed at pH 7.4, we refer to the X-ray analysis of the mutant Ala1b/F64L/Y66H/Q80R (Research Collaboratory for Structural Bioinformatics Protein Data Bank accession code 2emd).[13] The mutant chromophore with the Tyr66 residue of native GFP replaced by an imidazolyl group is planar and superimposes well on the chromophore of the wild-type protein. The smaller distal aromatic ring and its lack of a hydroxy group create a cavity, which is occupied by a water molecule. This water molecule apparently hydrogen bonds to the Nε2 atom of the chromophore, to the His148 residue, and to another water molecule (Scheme 1 B.) In contrast to the situation for the Y66F mutant, these



Scheme 1. *Hydrogen bonding of the distal aromatic ring of the chromophore in wild-type GFP (A) and the mutant Y66H (B) as taken from the X-ray structures.*[7, 13]

hydrogen bonds might raise the activation barrier for rotational motion of the distal aromatic ring of residue 66 and thus reduce internal conversion to favor emission by a factor of almost 13.

The following conclusions are derived from a qualitative analysis of the degrees of rotational freedom responsible for internal conversion in the mutant Y66H as compared to Y66F and wild-type GFP: 1) Internal conversion induced by a concerted and simultaneous rotation around both exocyclic C–C bonds[15] is not probable. This hula-twist motion would be rather insensitive to changes in how the distal aromatic ring is fixed, since both aromatic moieties of the chromophore are assumed to undergo only small amplitude motions. 2) Since fixation by hydrogen bonds has a comparatively small effect on the rate of internal conversion in the case of wild-type GFP (Scheme 1 A; see above), we conclude that rotation about the C_γ–C_β bond plays the central role in the internal conversion process. The type of fixation observed in wild-type GFP (Scheme 1 A) restricts only the rotational freedom about the C_β–C_α bond; rotation about the C_γ–C_β bond is hardly affected because of the rotational symmetry of the phenolic C–O bond which is in line with the axis of rotation. In contrast, the fixation in Y66H (Scheme 1 B) is likely to hinder rotation about both the C_β–C_α and the C_γ–C_β bonds. In this mutant the relevant hydrogen bond connects to the distal aromatic ring at a considerable angle with respect to the axis of rotation. Rotational motion coupled to internal conversion can thereby be efficiently suppressed, even though the free volume of the chromophore is enlarged.

Conclusions

Room-temperature picosecond time-resolved fluorescence decay traces of the GFP single-site mutants Y66F and Y66H, in which the tyrosyl residue of the chromophore is replaced, show excited-state lifetimes of 74 ps and 0.9 ns, respectively. When the temperature is lowered, these lifetimes increase and approach the radiative limit of 3 – 4 ns. The radiationless decay channel in these mutants is attributed to internal conversion favored by motional degrees of freedom around the exocyclic bonds of the chromophore. X-ray structural data on similar blue variants show specific changes in the hydrogen bond network and a slight increase of the free volume of the chromophore as compared to wild-type GFP.

The conclusion that internal conversion is reduced in the case of the smaller His66 chromophore relative to the Phe66 variant supports the notion that in GFP hydrogen bonding plays a more important role than tight packing in fixation of the chromophore. In wild-type GFP the phenolic oxygen is hydrogen bonded as well. However, the adjacent rotationally symmetrical C–O bond is in line with the proposed axis of rotation. This explains why the internal conversion rate in the protonated chromophore of wild-type GFP is only a factor of three smaller than in the Y66F mutant. Thus, wild-type GFP owes its high fluorescence quantum yield to extremely effective ultrafast excited-state proton transfer, which outpaces radiationless losses in the phenolic form and leads to the well-known green-emitting phenolate form of the chromophore with its long lifetime of 3.3 ns.

Materials and Methods

Protein expression, purification, and characterization:

The construction of the Y66F vector as well as protein expression (at 25 °C) and purification were performed as described previously.[22] The proteins were solubilized in phosphate buffered saline (KH_2PO_4 (4 mM), Na_2HPO_4 (16 mM), NaCl (115 mM), pH 7.4).

The Y66H variant was obtained by screening a randomly mutated library of GFP genes, constructed by mutagenic PCR of the complete wtGFP gene. A PCR mix (100 μL) contained tris(hydroxymethyl)aminomethane (10 mM, pH 8.3), bovine serum albumin (10 μg), KCl (50 mM), $MgCl_2$ (7 mM), guanosine triphosphate (2 mM), adenosine triphosphate (2 mM), thymidine triphosphate (10 mM), cytidine triphosphate (10 mM), each primer (3 nm), template DNA pt7GFav (42 ng), $MnCl_2$ (1.25 mM), and Taq polymerase (5 U). PCR primers were 5′-CGACTCACTATAGGGAGACCACAAC-3′ (forward) and 5′-GCTTCCTTTCGGGCTTTGTTAG-3′ (reverse). Taq polymerase and $MnCl_2$ were mixed shortly before they were added to the PCR mix. Cycling was performed with a paraffin overlay in a Landgraf thermocycler with 1 cycle at 94 °C for 60 s and 30 cycles at 94 °C for 60 s, 45 °C for 60 s, and 72 °C for 60 s. The PCR product was gel-purified, cleaved with NcoI and HindIII endonucleases, gel-purified again, and cloned back into the vector. *Escherischia coli* BL21 DE3 were electroplated with DNA and plated on ampicillin-containing Lennox broth (LB) agar. Screening of approximately 50 000 colonies was carried out with a handheld UV lamp (365 nm) in the dark. After four days, blue fluorescing colonies were found. The DNA was isolated, transformed in *E. coli* XL1 Blue, and prepared again. The presence of the Y66H single-site mutation among the selected genes was confirmed by DNA sequencing.

Spectroscopic measurements:

Steady-state absorption and fluorescence measurements: Steady-state absorption and fluorescence spectra were recorded with $\leq$ 2.0 nm resolution on a Perkin-Elmer Lambda 2S and a Spex Fluorolog-2 Model F212I spectrometer, respectively. A 1 mm-path-length quartz cuvette was used in both spectrometers and fluorescence was detected in a front face geometry. The sample cell was placed in a continuous-flow cryostat (Leybold VSK 3-300)

cooled with liquid nitrogen for both steady-state and time-resolved low-temperature experiments.

Picosecond time-resolved fluorescence measurements: Fluorescence kinetics were measured under magic angle conditions by using the time-correlated single-photon counting technique.[21] Standard deconvolution procedures were applied and averaged lifetimes shorter (down to a third) than the fwhm of the apparatus response function (in this instance 30 – 40 ps) were considered to have an ambiguity of a factor of 2. All other averaged lifetimes carry an approximate error of less than 25%.

This work was supported by the Deutsche Forschungsgemeinschaft within the frame of Sonderforschungsbereich 533.

[1] R. Y. Tsien, *Annu. Rev. Biochem.* **1998**, *67*, 509.
[2] A. B. Cubitt, R. Heim, S. R. Adams, A. E. Boyd, L. A. Gross, R. Y. Tsien, *Trends Biochem. Sci.* **1995**, *20*, 448.
[3] R. Heim, D. C. Prasher, R. Y. Tsien, *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 12501.
[4] a) F. Yang, L. G. Moss, G. N. Phillips, Jr., *Nat. Biotechnol.* **1996**, *14*, 1246; b) M. Ormö, A. B. Cubitt, K. Kallio, L. A. Gross, R. Y. Tsien, S. J. Remington, *Science* **1996**, *273*, 1392.
[5] M. Chattoraj, B. A. King, G. U. Bublitz, S. G. Boxer, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 8362.
[6] H. Lossau, A. Kummer, R. Heinecke, F. Pöllinger-Dammer, C. Kompa, G. Bieser, T. Jonsson, C. M. Silva, M. M. Yang, D. C. Youvan, M. E. Michel-Beyerle, *Chem. Phys.* **1996**, *213*, 1.
[7] K. Brejc, T. K. Sixma, P. A. Kitts, S. R. Kain, R. Y. Tsien, M. Ormö, S. J. Remington, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 2306.
[8] R. Heim, A. B. Cubitt, R. Y. Tsien, *Nature* **1995**, *373*, 663.
[9] R. Rizzuto, M. Brini, F. De Giorgi, R. Rossi, R. Heim, R. Y. Tsien, T. Pozzan, *Curr. Biol.* **1996**, *6*, 183.
[10] R. Heim, R. Y. Tsien, *Curr. Biol.* **1996**, *6*, 178.
[11] R. D. Mitra, C. M. Silva, D. C. Youvan, *Gene* **1996**, *173*, 13.
[12] G. J. Palm, A. Wlodawer, *Methods Enzymol.* **1999**, *302*, 378.
[13] G. J. Palm, A. Zdanov, G. A. Gaitanaris, R. Stauber, G. N. Pavlakis, A. Wlodawer, *Nat. Struct. Biol.* **1997**, *4*, 361.
[14] R. M. Wachter, B. A. King, R. Heim, K. Kallio, R. Y. Tsien, S. G. Boxer, S. J. Remington, *Biochemistry* **1997**, *36*, 9759.
[15] W. Weber, V. Helms, J. A. McCammon, P. W. Langhoff, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 6177.
[16] J. J. van Thor, A. J. Pierik, I. Nugteren-Roodzant, A. Xie, K. J. Hellingwerf, *Biochemistry* **1998**, *37*, 16915.
[17] S. Kojima, T. Hirano, H. Niwa, M. Ohashi, S. Inouye, F. I. Tsuji, *Tetrahedron Lett.* **1997**, *38*, 2875.
[18] A. A. Voityuk, A. D. Kummer, M. E. Michel-Beyerle, N. Rösch, *Chem. Phys.* **2001**, *269*, 83.
[19] This averaging procedure may overestimate the contribution of the longer time components in contrast to a stretched exponential treatment in which the amplitudes of the shorter components are more pronounced.
[20] A. D. Kummer, C. Kompa, H. Lossau, F. Pöllinger-Dammer, M. E. Michel-Beyerle, C. M. Silva, E. J. Bylina, W. J. Coleman, M. M. Yang, D. C. Youvan, *Chem. Phys.* **1998**, *237*, 183.
[21] A. D. Kummer, C. Kompa, H. Niwa, T. Hirano, S. Kojima, M. E. Michel-Beyerle, *J. Phys. Chem. B* **2002**, submitted.
[22] A. D. Kummer, J. Wiehler, H. Rehaber, C. Kompa, B. Steipe, M. E. Michel-Beyerle, *J. Phys. Chem. B* **2000**, *104*, 4791.

Received: December 20, 2001
Revised version: March 25, 2002 [F336]