Fluorescence Quenching of (Dimethylamino)naphthalene Dyes Badan and Prodan by Tryptophan in Cytochromes P450 and Micelles

Petr Pospíšil,† Katja E. Luxem,‡ Maräia Ener,‡ Jan Sýkora,‡ Jana Kocáblová,† Harry B. Gray,*‡ Antonín Vlček, Jr.*§ and Martin Hofbauer*‡

†J. Heyrovský Institute of Physical Chemistry, Academy of Sciences of the Czech Republic, Dolejškova 3, CZ-182 23 Prague, Czech Republic
‡Beckman Institute, California Institute of Technology, Pasadena, California 91125, United States
§School of Biological and Chemical Sciences, Queen Mary University of London, Mile End Road, London E1 4NS, United Kingdom

ABSTRACT: Fluorescence of 2-(N,N-dimethylamino)-6-propionyl-naphthalene dyes Badan and Prodan is quenched by tryptophan in Brij 58 micelles as well as in two cytochrome P450 proteins (CYP102, CYP119) with Badan covalently attached to a cysteine residue. Formation of nonemissive complexes between a dye molecule and tryptophan accounts for about 76% of the fluorescence intensity quenching in micelles, the rest is due to diffusive encounters. In the absence of tryptophan, fluorescence of Badan-labeled cytochromes decays with triexponential kinetics characterized by lifetimes of about 100 ps, 700–800 ps, and 3 ns. Site mutation of a histidine residue in the vicinity of the Badan label by tryptophan results in shortening of all three decay lifetimes. The relative amplitude of the fastest component increases at the expense of the two slower ones. The average quenching rate constants are 4.5 × 10⁸ s⁻¹ (CYP102) and 3.7 × 10⁸ s⁻¹ (CYP119), at 288 K. Cyclic voltammetry of Prodan in MeCN shows a reversible reduction peak at ~1.85 V vs NHE that becomes chemically irreversible and shifts positively upon addition of water. A quasireversible reduction at ~0.88 V was observed in an aqueous buffer (pH 7.3). The excited-state reduction potential of Prodan (and Badan) is estimated to vary from about +0.6 V (vs NHE) in polar aprotic media (MeCN) to approximately +1.6 V in water. Tryptophan quenching of Badan/Prodan fluorescence in CYPs and Brij 58 micelles is exergonic by ≤0.5 V and involves tryptophan oxidation by excited Badan/Prodan, coupled with a fast reaction between the reduced dye and water. Photo-reduction is a new quenching mechanism for 2-(N,N-dimethylamino)-6-propionyl-naphthalene dyes that are often used as solvatochromic polarity probes, FRET donors and acceptors, as well as reporters of solvation dynamics.

INTRODUCTION

Fluorescence quenching of organic dyes appended to proteins occurs either by energy transfer (FRET) or, less often, by electron transfer (ET).1,2 Quenching kinetics provide important information on substrate binding, conformational changes, intraprotein interactions, and protein folding, among others.3–7 FRET rates fall with the sixth power of the donor–acceptor distance and can be used to map relatively long-range interactions, from about 1 to 10 nm. On the other hand, the exponential decay of ET rates with distance8 allows investigating shorter-range interactions around 1 nm and below. Given the short inherent excited-state lifetimes of organic chromophores (typically 2–8 ns), only very fast ET leads to efficient fluorescence quenching. This usually happens only when the electron donor and acceptor come into a close contact, for example, by ππ stacking and/or hydrophobic forces9,10 Such interactions are then manifested by static fluorescence intensity quenching whereas fluorescence decay becomes multiexponential, showing very fast (usually tens of picosecond or less) kinetics component(s) due to emission from donor–acceptor contact complexes, and an unquenched decay due to residual free fluorophore.1,2 The on–off ET fluorescence switching upon emergence of a close donor–acceptor contact also can be detected by single-molecule techniques, such as fluorescence correlation spectroscopy, showing intensity fluctuations that report on protein conformational changes, folding, or on interaction dynamics, with applications in biomolecular recognition and molecular diagnostics.3,4,10,11 Fluorescence ET quenching in proteins has been studied mainly using oxazine, rhodamine, or Bodipy dye labels as excited-state electron acceptors (oxidants) and tryptophan (Trp) indole side chains as electron donors.3,4,10,11 Fluorescence of a variety of dyes also can be quenched by ET from a thioamide group incorporated into the peptide backbone, as has been employed to investigate protease activity and protein folding.12 Notably, electronically excited flavodox-
Ine cofactors are quenched by ET from Trp in photolyases and
cryptochromes, which are involved in DNA photorepair and
blue-light sensing by living organisms, respectively.\textsuperscript{13–16}

We have investigated fluorescence quenching of two 2-(N,N-
dimethylamino)-6-propionylnaphthalene dyes Badan and Pro-
dan to Trp in two cytochrome P450 mutants and in Brij 58
micelles (Figures 1 and 2). Fluorescence of dimethylnaph-
thalene-based dyes originates from an intramolecular charge-
transfer excited state in which electron density is transferred
from the NMe\textsubscript{2} group to the electron-accepting carbonyl
substituent at the naphthalene 6-position.\textsuperscript{1,2,17–19} These
photophysics are strongly medium-dependent, presumably
due to the molecular dipole moment increasing upon excitation
and specific solvation, including hydrogen bonding.\textsuperscript{2,19} The
dyes are frequently used as probes of polarity and dynamics of
the local environment in proteins\textsuperscript{1,2,17,18} as well as micelles and
membranes,\textsuperscript{1,2} by measuring stationary and time-resolved
fluorescence spectra.\textsuperscript{1} The environmental dependence of Badan
fluorescence lifetimes was employed, for example, in a glucose
sensor.\textsuperscript{22} These utilities prompted the development of
dimethylnaphthalene-based dyes with a broad range of protein
labeling groups, including Badan (thiol-reactive bromine,
Figure 1), Acrylodan (cysteine-reactive ethylene), the unnatural
amino acid Aladan for incorporation into peptide chains,
Danka, which binds apomyoglobin in a single orientation
through a carbonyl group, and Dansyl, where the 6-
propionyl is replaced by a sulfonamide. Prodan (and its
derivatives with long aliphatic chains) are typically used as
noncovalent membrane probes.\textsuperscript{1,17}

(Dimethylamino)naphthalene-type dyes also serve as energy
acceptors from electronically excited tryptophan, *Trp,\textsuperscript{1,23} or as
energy donors toward hemes in cytochromes,\textsuperscript{24,25} as utilized in
FRET experiments. Photoinduced electron transfer, in contrast,
is not a common quenching mechanism of these dyes, although
Badan photo reduction by Trp was proposed to occur in one of
the CYP3A4 conformers.\textsuperscript{5} An opposite process, photooxidation
of a (dimethylamino)naphthalene by a guanine cation, has been
observed in a protein–DNA complex.\textsuperscript{26}

Herein, we present compelling evidence for ET quenching of
Badan fluorescence by comparing the behaviors of Badan-
labeled cytochrome P450 mutants (CYP, Figure 2) with and
without a Trp residue in the dye vicinity. The occurrence of
photoinduced ET from Trp to electronically excited 2-
(dimethylamino)-6-propionylnaphthalene dyes is further sup-
ported by electrochemistry and by fluorescence quenching of
Badan and Prodan in Trp-containing micelles. Observation of
an ET quenching mechanism for these dyes highlights an
important constraint for their use as microenvironmental
probes, while opening new avenues for study of ultrafast
dynamics associated with short-range interactions in biological
systems.

\section*{EXPERIMENTAL SECTION}

\textbf{Materials.} Badan and Prodan were obtained from Anaspec
and Invitrogen, respectively, and used without further purification. Brij 58 and tryptophan were obtained from Sigma-Aldrich.

\textbf{Protein Expression.} The CYP102 (also called P450 BM3 or
CYP102(A1)) C62A/C156S/K97C triple mutant has been

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Prodan and Badan dye molecules (top) and the Brij 58
detergent molecule.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Schematic structures and abbreviations of the investigated Badan-labeled cytochrome P450 mutants \textit{Bacillus megaterium} CYP102 (top, also
known as P450 BM3 or CYP102(A1)) and \textit{Sulfolobus acidocaldarius}
CYP119 (bottom). Badan (yellow) is attached to Cys97 and Cys81, respectively.
Native residues Trp96 of CYP102 and His76 of CYP119 (middle panels) are replaced by His96 and Trp76, respectively, in the swap mutants (right
panels). The heme is shown in red, His in orange, Trp in blue, and Cys and Badan in yellow. Based on unlabeled protein structures: pdb 2IJJ\textsuperscript{27}
(CYP102-Trp), pdb 1IO7\textsuperscript{28} (CYP119-His), and yet unpublished structure of CYP102-His. The Badan orientation was chosen arbitrarily for
illustrative purposes.}
\end{figure}
reported. The His-containing mutant C62A/C156S/K97C/W96H and CYP119 mutants S81C/H86 and S81C/H76W were prepared by the same procedure. The plasmid for CYP119 was obtained from Prof. Paul Ortiz de Montellano (UCSF). All mutations were made using aQuickChange Site-Directed Mutagenesis kit (Qiagen). All primers were obtained from Operon. The proteins were expressed with an N-terminal His
tag in Escherichia coli BL21(DE3) cells as described previously. All proteins were purified according to a literature procedure and characterized by ESI mass spectrometry.

**Protein Labeling.** Approximately a 5-fold molar excess of Badan was added to a 10 μM solution of protein in 20 mM Tris buffer (pH 8), and the mixture was shaken in the dark at 4 °C for 4 h. Excess Badan was removed during concentration in 30 kDa filters, and the protein was purified on a PD-10 desalting column (GE Healthcare and Life Sciences). Mass spectrometry indicated that the samples were labeled to 75–100%. Concentrated protein samples were stored with dithiothreitol (DTT, to prevent disulfide bond formation) in 25% glycerol solutions at −80 °C. Prior to use, they were thawed on ice and DTT was removed using a HiTrap desalting column (GE Healthcare Bio-Science AB, Uppsala) in a 20 mM Tris (pH 8) buffer. Samples were stored in the dark at 4 °C.

**Preparation of Badan/Tryptophan-Containing Micelle Solutions.** Badan (or Prodan) was added in a 1:2000 ratio to Solutions. Badan was added to a 10 nM solution of the detergent Brij 58 in 20 mM Tris buffer (pH 8). The Brij 58 concentration was kept 2 orders of magnitude above the critical micelle concentration (CMMC ∼ a S). The solutions at (DTT, to prevent disulfide bond formation) in 25% glycerol solutions at −80 °C. Prior to use, they were thawed on ice and DTT was removed using a HiTrap desalting column (GE Healthcare Bio-Science AB, Uppsala) in a 20 mM Tris (pH 8) buffer. Samples were stored in the dark at 4 °C.

**Instrumentation.** Stationary emission spectra were obtained on a Fluorolog-3 spectrophotometer (model FL3-11; HORIBA Jobin Yvon) equipped with a Xenon-arc lamp. All spectra were collected in 1 nm steps (2 nm bandwidths were chosen for both the excitation and emission monochromators). Time-resolved fluorescence decays were measured using the time-correlated single photon counting technique on an IBH 5000 U SPC instrument equipped with a cooled Hamamatsu R3809U-50 microchannel plate photomultiplier with 40 ps time resolution and time setting of 7 or 14 ps per channel. Bandwidths for both the excitation and emission monochromators were set to 16 nm. To eliminate scattered light, a 399 nm cutoff filter was used. Samples were excited at 373 nm with an IBH NanoLED-11 diode laser (80 ps fwhm) with a repetition frequency of 1 MHz. The detected signal was kept below 20 000 counts per second to avoid shortening of the recorded lifetime due to the pile-up effect. Fluorescence decays were fitted (using the iterative reconvolution procedure with IBH DAS6 software) to a multiexponential function (eq 1) convoluted with the experimental response function IRF (‘prompt’), yielding sets of lifetimes \(τ\) and corresponding amplitudes \(A_i\). The average lifetimes \(⟨τ⟩\) were calculated according to eq 2.

\[
I(t) = \sum_i A_i e^{-t/τ} \otimes \text{IRF}
\]

\[
⟨τ⟩ = \frac{\sum_i A_i τ_i^2}{\sum_i A_i τ_i}
\]

We also report relative lifetime-weighted amplitudes \(B_i\) that are proportional to the relative number of photons emitted in a kinetics component \(i\) (eq 3).

\[
B_i = \frac{A_i τ_i}{\sum_i A_i τ_i} \times 100
\]

**Electrochemistry.** Cyclic voltammograms were obtained in MeCN containing 0.1 M Bu4NPF6 as an electrolyte and in aqueous Britton-Robinson buffer (pH = 7.3, I = 90 mM), using a three-electrode electrochemical cell with a hanging Hg drop working electrode, a platinum mesh auxiliary electrode and Ag/AgCl/1 M LiCl reference electrode, which was separated from the test solution by a salt bridge with two frit junctions and whose potential (−90 mV vs NHE) was calibrated using the Fe+/Fe couple as an internal standard (+0.54 V at a Pt working electrode vs Ag/AgCl/1 M LiCl; +0.630 V vs NHE). All potentials are reported vs NHE.

**RESULTS**

**Fluorescence Quenching in Badan-Labeled CYP Mutants.** All four investigated CYPs show a typical Badan fluorescence band at about 485 nm using excitation at 373 nm (Figure S1, Supporting Information). Fluorescence decay was investigated at 550 nm, within the red side of the emission band. (The emission wavelength of 550 nm was chosen to avoid interference from fast decay components due to medium relaxation instead of population decay. These relaxation dynamics are currently under investigation in our laboratories.) Figure 3 shows that fluorescence decays of CYP102-Trp and CYP119-Trp were faster than in the case of their His counterparts.

Emission decay kinetics were multieponential for all mutants, regardless whether Trp or His was present. Fitting to a triple exponential function yielded kinetics components occurring in the tens-of-picosecond (\(τ_1\)), hundreds-of-picosecond (\(τ_2\)), and nanosecond (\(τ_3\)) ranges (Table 1). Substituting His by Trp shortened all three lifetimes and strongly increased the relative amplitude of the fastest process, \(A_1\) and \(B_1\), indicating that the excited-state quenching reactions occur in several parallel pathways with lifetimes ranging from tens of picoseconds to tens of nanoseconds. The average quenching rate constants \(k_q\) (eq 4) are comparable for CYP102-Trp and CYP119-Trp (\(4 \times 10^8 \text{ s}^{-1} \text{ at } 288 \text{ K})\), whereas the quenching rate constant \(k_q3\) (based on the slow kinetics component \(τ_3\)) is 1.4–1.6 times higher for CYP102-Trp, in accordance with a shorter Trp–Badan distance (Figure 2). As expected, both quenching rate constants slightly decreased on decreasing the temperature from 296 to 288 K.
Badan Fluorescence Quenching in Micelles. Addition of a large excess of the surfactant Brij 58 to an aqueous Badan solution (Tris, pH 8) strongly increased Badan solubility and shifted the fluorescence maximum from 550 to 500 nm (Figure S2, Supporting Information), indicating that the micellar environment is a good model for the protein (λ_em = 485 nm). Prodan showed very similar behavior. Badan fluorescence decayed single-exponentially, τ_0 = 2.64 ns, whereas Prodan showed a small initial 0.55 ns rise followed by a 3.87 ns decay (all measurements at 550 nm). The lifetime-weighted rise amplitude was less than 10% of the decay amplitude. The rise component is tentatively attributed to a dynamic Stokes shift, whereby the fluorescence intensity initially increases in the red part of the band.2,17,32

 Addition of Trp quenched fluorescence intensity more strongly than the lifetime (Figures 4 and S2, Tables S1 and S2, Supporting Information). This behavior is characteristic of combined dynamic and static quenching,1,2 which is described by Stern–Volmer (SV) equations (eq S and 6).

\[
\frac{\tau}{\tau_0} = 1 + k_2\tau_0[\text{Trp}]
\]

Here, \(k_2\) is the bimolecular rate constant corresponding to dynamic (collisional) quenching and \(K\) is the stability constant of a supposedly nonemissive Trp–Badan (or Trp–Prodan) complex. The assumption of an “instantaneous” quenching upon a close contact between the dye and Trp is supported by the lack of any observable ultrafast decay kinetics, indicating that the excited-state lifetime of any such complex is shorter than 50 ps. Analysis of the lifetime quenching yielded a \(k_2\) value of \(1.74 \times 10^9\) M\(^{-1}\) s\(^{-1}\) for Badan and \(5.79 \times 10^8\) M\(^{-1}\) s\(^{-1}\) for Prodan (based on \(r_{\text{dec}}\) values listed in Table S2, Supporting Information). Lifetime quenching kinetics are virtually independent of the emission wavelength, as was checked for Badan at 500 nm (\(k_2 = 1.90 \times 10^9\) M\(^{-1}\) s\(^{-1}\)) and Prodan at 490 nm (\(5.74 \times 10^8\) M\(^{-1}\) s\(^{-1}\)). Intensity-based SV plots (Figure 4) yielded (after correction for dynamic quenching) the bimolecular static quenching constant \(K\) values of 14.7 and 3.5 M\(^{-1}\) for Badan and Prodan, respectively. The percentage of the total intensity quenching due to static quenching was estimated as 100%(\(K\) + \(k_2\tau_0\)) ~76% for Badan and ~61% for Prodan. Stationary fluorescence spectra also showed a very small blue shift upon Trp addition, from 509 to 504 nm, with an isoemissive point at 484 nm (Figure S3, Supporting Information).

Table 1. Fluorescence Decay Kinetics of Badan-Labeled CYP Mutants

| sample   | T (K) | lifetimes (ns) | \(r\)-weighted amplitudes (%) | amplitudes (%) | average lifetime (ns)\(^a\) | \(k_q\) (s\(^{-1}\)) |
|----------|-------|----------------|-----------------------------|----------------|-----------------------------|------------------|
|          |       | \(\tau_1\) | \(\tau_2\) | \(\tau_3\) | \(A_1\) | \(A_2\) | \(A_3\) | \(A_4\) | \(k_2\tau_0\) | \(k_q\) |
| CYP102-Trp | 288   | 0.02 \(^c\) | 0.14 | 0.24 | 33 | 37 | 30 | 96 | 3 | 1 | 0.86 | 4.5 \times 10^9 | 1.0 \times 10^9 |
| CYP102-His | 288   | 0.13 | 0.14 | 0.24 | 19 | 46 | 35 | 66 | 28 | 6 | 1.40 | 5.1 \times 10^9 | 1.4 \times 10^9 |
| CYP102-Trp | 296   | 0.02 \(^c\) | 0.10 | 0.24 | 33 | 39 | 39 | 96 | 3 | 1 | 0.95 | 3.7 \times 10^8 | 7.3 \times 10^7 |
| CYP102-His | 296   | 0.09 | 0.10 | 0.30 | 12 | 36 | 52 | 65 | 26 | 9 | 1.85 | 3.8 \times 10^8 | 8.8 \times 10^7 |
| CYP119-Trp | 288   | 0.05 | 0.10 | 0.24 | 30 | 40 | 30 | 87 | 11 | 2 | 0.90 | 3.8 \times 10^8 | 8.8 \times 10^7 |
| CYP119-His | 288   | 0.10 | 0.19 | 0.28 | 17 | 45 | 38 | 68 | 26 | 6 | 1.35 | 3.8 \times 10^8 | 8.8 \times 10^7 |
| CYP119-Trp | 296   | 0.05 | 0.20 | 0.30 | 31 | 40 | 29 | 88 | 10 | 2 | 0.94 | 3.8 \times 10^8 | 8.8 \times 10^7 |
| CYP119-His | 296   | 0.10 | 0.20 | 0.30 | 19 | 45 | 35 | 72 | 23 | 5 | 1.47 | 3.8 \times 10^8 | 8.8 \times 10^7 |

\(^a\)The \(\tau_1\) kinetics occur at the limit of the experimental time resolution, and the corresponding lifetime and amplitude values were obtained by IRF deconvolution. Although this might introduce some absolute error, it did not affect significantly the relative change between His- and Trp-containing mutants. \(^b\)Virtual identical average lifetime values were obtained from 4-exponential fits. Accuracy ±50 ps. \(^c\)Estimated, at the limit of IRF deconvolution.
Information). This effect indicates the presence of at least two slightly different Badan populations in the micelles, the red-absorbing one being preferentially quenched by Trp.

**Electrochemistry.** 2-(N,N-Dimethylamino)naphthalene dyes are known to undergo irreversible electrochemical oxidation that was studied in detail for Prodan. Herein, we report on its reductive electrochemistry that is relevant to the fluorescence quenching by Trp. A cyclic voltammogram (CV) of Prodan in MeCN (Figure 5) showed a nearly reversible reduction at $E_{1/2} = −1.85$ V vs NHE. The small prewave is due to adsorption of the reduced form. Changing the solvent from MeCN ($\epsilon = 35.9$) to a more polar DMSO ($\epsilon = 46.5$) has only a small effect: the reduction wave stays reversible and shifts to $−1.77$ V. No reduction is observed in much less polar 1,2-dichloroethane ($\epsilon = 10.4$) up to $−2.2$ V. On the other hand, water changes the reduction mechanism and shifts the potential positively: A small addition of water (1%) to the solution of Prodan in MeCN made the reduction chemically irreversible and shifted the cathodic peak potential ($E_{\text{pc}}$) by $+70$ mV while two small anodic peaks appeared at $−0.25$ and $−0.48$ V, attributable to reoxidation of the decomposition products of reduced Prodan. CV in MeCN containing $17\%$ H$_2$O showed a chemically irreversible reduction peak whose $E_{\text{pc}}$ is shifted by $+280$ mV relative to neat MeCN. The peak shape is consistent with rapid heterogeneous electron transfer, followed by a fast reaction with H$_2$O (presumably protonation). The CV in H$_2$O (Britton–Robinson buffer, pH 7.3) showed a quasi-reversible peak at $−0.88$ V ($E_{\text{p1}} − E_{\text{pc1}} = 0.18$ V) followed by two chemically irreversible reduction peaks at $−1.24$ and $−1.40$ V. The signal was very weak due to low Prodan solubility. (Results obtained for Prodan should be extendable to Badan because both species contain the same photo- and electroactive group, i.e., 2-(N,N-dimethylamino)-6-propionynaphthalene (Figure 1). However, Badan electrochemistry was complicated by the presence of a reducible C–Br bond.)

The excited-state reduction potential of Prodan and Badan in MeCN can be estimated as a sum of the ground-state reduction potential ($−1.85$ V) and the excited-state energy of $2.5$ eV ($E_{\text{iso}}$ estimated from fluorescence spectra), giving the value of $+0.6$ V vs NHE. Generally, the excited-state redox potential will depend on medium polarity that affects both the ground-state reduction potential and the excited-state energy. However, these two effects could partly compensate each other as decreasing medium polarity increases $E_{\text{iso}}$ while shifting the reduction potential negatively. Both dyes become much stronger excited-state oxidants (ca. $+1.6$ V) in the presence of water, as the reduced form is stabilized by a very rapid reaction with the solvent. The actual value depends on the water accessibility of the dye molecules.

### DISCUSSION

Fluorescence lifetimes of the two investigated Badan-labeled CYP-Trp mutants were significantly shorter than those of their His-containing counterparts, indicating that the emissive singlet excited state of Badan is quenched by the proximal Trp residue. To study the Badan−Trp quenching without any possible interfering effects of the protein environment or the heme cofactor, we also investigated Badan (and analogous Prodan) fluorescence quenching by Trp in Brij S8 micelles. Both species concentrate in the micelles, promoting formation of contact pairs. Indeed, we observed very efficient static quenching attributable to the formation of nonemissive ππ complexes between the aromatic Trp-indole groups and Badan (Prodan) inside micelles. These complexes are moderately stable, with association constants of $14.7$ M$^{-1}$ (3.5 M$^{-1}$), and their excited-state lifetimes are much shorter than the instrument time resolution, much less than 50 ps. Residual dynamic quenching, which accounts for $25$–$40\%$ of the total quenching, is attributable to the highly dynamic nature of the micelles that still allows for diffusive encounters.

Quenching of Badan and Prodan fluorescence can occur by either an energy- or electron-transfer mechanism. Energy transfer can be ruled out, because the population of *Trp* is energetically uphill (and *3Trp* is slightly uphill and spin-forbidden). The feasibility of an electron-transfer mechanism, i.e., Trp oxidation by excited dyes, can be assessed using electrochemical arguments. Trp is a redox-active amino acid whose indole side chain is oxidized to the corresponding radical cation at rather positive potentials, between $+1.02$ and $+1.21$ V vs NHE. Above, we have estimated the excited-state reduction potential of Prodan and Badan as $+0.6$ V in aprotic media and $+1.6$ V in water. It follows that electron-transfer quenching in aprotic media is endergonic by about 0.5 V (taking $E_{\text{CT}}^{\ast} (\text{Trp}/\text{Trp}^{\ast}) = +1.1$ V$^{35}$). In CYPs, Badan is located at the protein surface, partly exposed to water (Figure 2). In Brij S8 micelles, the dye and Trp molecules likely occur in the regions between the polyether head groups that are intermixed with water. It is the aqueous solvation that makes the photoquenching of (dimethylamino)naphthalene dyes by Trp thermodynamically possible because water changes the dye reduction mechanism and upshifts the excited-state reduction potential to roughly $+1.6$ V (the actual value likely depends on the dye exposure to water). Trp oxidation by electronically excited Badan (Prodan) in the present systems is thus thermodynamically favorable by $≤0.5$ V and ET is coupled to a very fast reaction between the solvating water molecules to the reduced dye (presumably proton transfer).

Having established Badan photoreduction by Trp as the most likely quenching mechanism, we turn our attention to the fluorescence decay kinetics of Badan-labeled CYPs. Both CYP102-His and CYP119-His exhibited multiplexponential behavior that probably reflects conformational heterogeneity, together with medium relaxation dynamics in the Badan vicinity. The longest lifetime measured for the His-containing mutants ($\tau_p ≃ 3$ ns) is longer than that observed in aqueous...
solutions (1.3–1.4 ns, Prodan)\(^4\) or in Brij micelles (2.64 ns, Table S1, Supporting Information), indicating that the Badan label is to some extent shielded from the solvent and oriented to disfavor energy transfer to the heme. The two faster components likely arise from conformations where Badan is more exposed and/or quenched by Förster energy transfer to the heme. (Indeed, heme cofactors are known to quench fluorescence of (dimethylamino)naphthalene dyes, such as Dansyl.)\(^5\) Crystal structures of unlabeled CYP102-Trp (pdb 2I2J) and CYP102-His (to be published) show that the five-membered part of the Trp96 indole ring of CYP102-Trp overlaps perfectly with the imidazole ring of the His mutant. The distances and orientations of the heme also are comparable. In both mutants, the Badan-bearing Cys97 is, on average, 6 Å away from the aromatic side chain (His96 or Trp96). The structure also reveals the presence of various rotamers that introduces significant variability in positions and orientations of the Cys97 sulfur atom and, hence, of the attached Badan label. The similarity between the structures of the Trp- and His-containing mutants supports our conclusion that the fluorescence lifetime decrease on going from CYP102-His to CYP102-Trp is attributable solely to the photoinduced ET between excited Badan and Trp. (Presumably, this conclusion remains valid also for CYP119, where only the structure of the His mutant is available, pdb 1I07.) In particular, replacing the His residue proximal to Badan by Trp caused quenching rate constant of about 4 × 10^8 M^−1 s^−1 for both CYP102-Trp and CYP119-Trp (at 288 K). Quenching influences all three fluorescence decay components (Table 1). The most pronounced change was observed for the shortest, tens-of-picoseconds, component that became much shorter lived and whose relative amplitude strongly increased. This fastest quenching process presumably occurs in a protein conformation where the Trp-indole and Badan make a close contact, resulting in almost complete quenching. The amplitude increase then reflects a change in conformational distribution, whereby close Trp–Badan contact formation is driven by ππ and/or hydrophobic interactions. Quenching of the longest lifetime τ\(_3\) (k\(_{q,3}\) = 1 × 10^8 and 7 × 10^7 s^−1 for CYP102 and CYP119, respectively) is too slow for a contact pair. Instead, it probably corresponds to long-range ET from Trp to the excited Badan label.

We conclude that (dimethylamino)naphthalene-based dyes Badan and Prodan can behave as photooxidants whose excited-state reduction potentials vary from about +0.6 V (vs NHE) in polar aprotic media (MeCN) to approximately +1.6 V in water, where reduction is presumably coupled to proton transfer. Fluorescence of these (dimethylamino)naphthalene dyes is quenched by close-lying tryptophan in proteins as well as micelles. Excited-state ET reactions in Badan-labeled CYPs occur in several kinetics steps whose lifetimes range from tens of picoseconds to about 10 ns, presumably depending on protein conformation as well as the relative orientation of the Badan and indole aromatic groups. The possibility of photoinduced ET must be considered whenever (dimethylamino)naphthalene dyes are used as fluorescence protein labels, as this reaction channel can complicate the interpretation of FRET experiments as well as analyses of solvation dynamics studied by time-resolved fluorescence spectroscopy. On the other hand, as nanosecond Badan fluorescence quenching kinetics are expected to be sensitive to structural and conformational factors, Badan protein labeling could be employed to investigate short-range (≤10 Å) intraprotein interactions and conformational changes due to folding or substrate binding.

### REFERENCES

(1) Lakowicz, J. R. Principles of Fluorescence Spectroscopy, 3rd ed.; Springer: New York, 2006.
(2) Valeur, B. Molecular Fluorescence. Principles and Applications; Wiley-VCH: Weinheim, 2002.
(3) Doose, S.; Neuweiler, H.; Sauer, M. Fluorescence Quenching by Photoinduced Electron Transfer: A Reporter for Conformational Dynamics of Macromolecules. ChemPhysChem 2009, 10, 1389–1398.
(4) Doose, S.; Neuweiler, H.; Sauer, M.; Close, A. Look at Fluorescence Quenching of Organic Dyes by Tryptophan. ChemPhysChem 2005, 6, 2277–2285.
(5) Tsalkova, T. N.; Davydova, N. Y.; Halpert, J. R.; Davydov, D. R. Mechanism of Interactions of R-Naphthoflavone with Cytochrome P450 3A4 Explored with an Engineered Enzyme Bearing a Fluorescent Probe. Biochemistry 2007, 46, 106–119.
(6) Dunn, A. R.; Hays, A.-M. A.; Goodin, D. B.; Stout, C. D.; Chiu, R.; Winkler, J. R.; Gray, H. B. Fluorescent Probes for Cytochrome P450 Structural Characterization and Inhibitor Screening. J. Am. Chem. Soc. 2002, 124, 10254–10255.
(7) Holt, A.; Koehorst, R. B. M.; Rutters-Meijneke, T.; Gelb, M. H.; Rijkers, D. T. S.; Hemmings, M. A.; Killian, J. A. Tilt and Rotation Angles of a Transmembrane Model Peptide as Studied by Fluorescence Spectroscopy. Biophys. J. 2009, 97, 2258–2266.
(8) Gray, H. B.; Winkler, J. R. Long-Range Electron Transfer. Proc. Natl. Acad. Sci. U. S. A. 2005, 102, 3534–3539.
(9) Vaiana, A. C.; Neuweiler, H.; Schulz, A.; Wolfrum, J.; Sauer, M.; Smith, J. C. Fluorescence Quenching of Dyes by Tryptophan: Interactions at Atomic Detail from Combination of Experiment and Computer Simulation. J. Am. Chem. Soc. 2003, 125, 14564–14572.
(10) Neuweiler, H.; Johnson, C. M.; Fersht, A. R. Direct Observation of Ultrafast Folding and Denatured State Dynamics in Single Protein Molecules. Proc. Natl. Acad. Sci. U. S. A. 2009, 106, 18569–18574.
(11) Neuweiler, H.; Banachewicz, W.; Fersht, A. R. Kinetics of Chain Motions Within a Protein-Folding Intermediate. Proc. Natl. Acad. Sci. U. S. A. 2010, 107, 22106–22110.
