Use of Thiouredopyrenetrisulfonate Photochemistry for Driving Electron Transfer Reactions in Aqueous Solutions†

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ABSTRACT: Photoexcitation of 1-thiouredopyrene-3,6,8-trisulfonic adducts (TUPS) of amino acids by the third harmonic frequency of a Nd:YAG laser (355 nm) generates the triplet state of the dye with high quantum efficiency. Relaxation of the triplet proceeds in anaerobiosis with a half decay time of 0.5 ms. The relaxation rate increases 100-fold in the presence of dioxygen. A radiative transition between the triplet and the ground state of the dye results in phosphorescent emission centered at 658 nm. The excited state of TUPS, being a strong reductant, can donate its electron to a variety of acceptors. Transient absorption spectroscopy was used to directly measure the photoinduced electron transfer from the excited dye to rhodamine B (RB) and cytochrome *c*. The reaction with RB was followed by monitoring the oxidation of the triplet state of TUPS at 487 nm ($\epsilon = 25\,000 \pm 5\,000$ M^{-1} cm^{-1}) or the reduction of RB at 553 nm. The second order rate constant for the reaction was found to be $(2.5 \pm 0.2) \times 10^9$ M^{-1} s^{-1}, a value compatible with that for diffusion controlled reactions. When directed to cytochrome c the photoinduced perturbation causes rapid reduction of the protein's heme group, seen as a monophasic increase of absorbance at 550 nm. The combination of appropriate redox properties with the capability of covalent protein modification makes the dye useful for initiation and analysis of electron transfer reactions in chemical and biological systems.

Inter- and intraprotein electron transfer processes are key steps in biological energy conservation systems. While steady state kinetics is readily used for the measurement of electron transport between the redox carriers, time-resolved measurements have until recently been limited to photosynthetic systems, where photochemical activation initiates electron transfer allowing the resolution of nanosecond and microsecond electron transfer dynamics (*1*,*2*).

The inability of non-photosynthetic systems to interact with radiation can be circumvented by using external photosensitive elements as electron donors. Current experimental methods employ triplet states of photoexcited flavins (*3*,*4*) and ruthenium complexes (*5*,*6*) or zinc-substituted heme (*7*,*8*) as light-driven photoperturbants. Of these, the ruthenium (Ru) complexes have proven to be the most practical. Pulse irradiation of Ru(II) complexes results in the formation of a low potential triplet state ($E° = -0.72$ eV). Upon relaxation of the oxidized metal ion to the ground state, Ru(III) acts as an oxidant ($E° = +1.31$ eV) and becomes re-reduced.

We have recently introduced sulfoaromatic compounds for the perturbation of redox equilibrium in aqueous solutions (*9*). Under a high photon flux (50 MW/cm^2), these compounds undergo a fast photoxidation: a successive two-photon reaction ejects an electron from the molecule and a pair of reactants: a solvated electron and an oxidized aromatic moiety are formed. Under conditions where the solvated electrons are quenched, it is a stable compound that reacts as a one-electron acceptor with a variety of reductants.

In the present study we have expanded the applicability of sulfoaromatic compounds for the study of electron transfer in proteins by the introduction of IPTS,[1] capable of specific interaction with protein amino groups. We have shown that at low (5 MW/cm^2) excitation power the thiouredo adduct (TUPS), formed by the reaction of the dye with free amino acids, is excited by a single photon to the triplet state (TUPS*). High yield, long life time, and low redox potential of the triplet state make it an efficient initiator of electron transfer processes. The redox properties of the dye are studied, and its utilization in driving redox perturbation in proteins is discussed.

MATERIALS AND METHODS

Materials. IPTS was purchased from Lambda Fluorescence (Austria). $K_4(Fe(CN)_6)$ AR is a Merck product. All other chemicals were obtained from Sigma (U.S.A.).

Preparation of Amino Acid−TUPS Derivatives. TUPS−amino acid derivatives were prepared by incubation of IPTS with free amino acids. Amino acids (5 mM) in 50 mM Hepes/KOH (pH 8.5) were mixed with freshly prepared IPTS (1 mM) and incubated for 15 h at 38 °C. The TUPS−amino acid complexes were separated from nonbound IPTS and its hydrolyzed forms at room temperature on a LiChrosorb RP-18, 50-A reverse-phase HPLC column (250 × 4.6 mm, from Knalier, Germany). The dye-containing peaks were eluted at a flow rate of 0.7 mL/min by a linear gradient of acetonitrile from 4 to 50 % (0.1% TFA). The gradient was generated on a dual pump system (model 626), and the elution was monitored at 372 nm on a UV/visible detector (model PDA 996) both from Waters. The peaks, containing TUPS−amino acid derivatives, were collected. The best

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[1] Abbreviations: IPTS, 1-isothiocyanatopyrene-3,6,8-trisulfonate; TUPS, thiouredopyrene-3,6,8-trisulfonate; TUPS*, excited triplet state of thiouredopyrene-3,6,8-trisulfonate; RB, rhodamine B.

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resolution was obtained with TUPS−Leu and TUPS−Val derivatives.

Optical and Luminescent Measurements. Optical absorption spectra were measured with a Hitachi model U-2000 spectrophotometer. Fluorescence and phosphorescence measurements were conducted on a Perkin Elmer model LS50B luminescence spectrometer. Phosphorescence was measured in anaerobic conditions. To remove oxygen dissolved in the solution glucose oxidase (10 μg/mL), catalase (1 μg/mL) and glucose (20 mM) were added to the assay. The photons emitted were counted during a 10 ms period, starting 0.1 ms after the 372 nm excitation flash.

Transient Absorption Measurements. The sample (1.5 mL) containing 5 mM Hepes/KOH (pH 7.0) and TUPS−amino acid derivative (20 μM) was placed in a four-face 1 cm quartz cuvette. The oxygen dissolved in the solution was removed by continuous bubbling of Ar at 15 mL/min through the cuvette for 15 min and throughout the experiment or by a glucose oxidase-catalase system. Excitation of the dyes was initiated by the third harmonic frequency of a Nd:YAG laser (355 nm, 2 ns full-width at half-maximum, 3 mJ/pulse), which was focused on the side of the cuvette over a spot having a surface of 0.3 cm^2. Continuous monitoring of the redox state of the amino acid−TUPS adducts was carried out at 487 and 458 nm The redox states of the cytochrome's heme group and RB were monitored at 550 minus 556 nm (the reference 556 nm wavelength is an isosbestic point of cytochrome *c*) and 553 nm, respectively. The probing beams, 458 and 487 nm generated by a CW argon laser or 550, 556, and 553 nm generated by a "Coherent" CR-599 dye laser with rhodamine-110 dye, were perpendicular to the pulse-irradiated face of the cuvette. The probing beam was directed to a monochromator photomultiplier assembly, and transients were stored and averaged by a Tektronix TDS 520A digital oscilloscope as previously described (*9*). The response time of the detection system is 20 ns. The transients are the average of 20 pulses collected at a frequency of 0.02 Hz. Reduction of cytochrome *c* was quantitated by the absorbance coefficient difference of $\epsilon_{550-556} = 19\,000$ M^{-1} cm^{-1} and the reduction of RB by absorbance coefficient of $\epsilon_{553} = 108\,000$ M^{-1} cm^{-1} (*10*).

RESULTS

Spectroscopic Characterization of the Dye. The dyes based on pyrenyloxytrisulfonate acid (Cascade Blue) are known as fluorescent protein labels (*11*). Despite their acceptance as fluorescent tracers, very few studies (*11*, *12*) describing the use of these dyes in protein research are available. The absorption and fluorescent properties of IPTS and TUPS molecules have not been systematically studied either. The absorption spectrum of a fresh aqueous solution of IPTS has a maximum at 401 nm (see Figure 1A). The luminescence emission spectrum of IPTS has its maximum centered around 420 nm (see Figure 1B). Both optical and luminescent properties of IPTS are characteristic of Cascade Blue dyes. Conjugation with amino acids, the reaction that yields the thiouredo form of the dye (TUPS), strongly alters its optical and fluorescent properties. The absorption spectrum of the thiouredo adduct is ∼30 nm blue-shifted, and the fluorescence of the dye is strongly quenched (see Figure 1). The decay in fluorescence intensity is accompanied by a rise of phosphorescence. The thiouredo

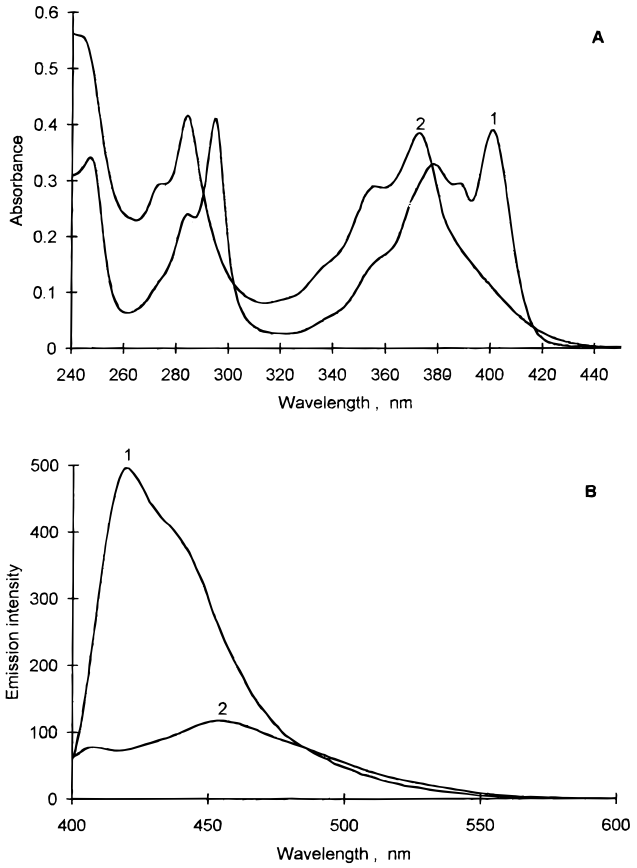

FIGURE 1: Absorption and fluorescent spectra of IPTS and TUPS. (A) Absorption spectra of freshly prepared 10 μM IPTS (curve 1) and 10 μM Leu−TUPS (curve 2) in 5 mM Hepes/KOH (pH 7.0). (B) Emission spectra of freshly prepared 1 μM IPTS (curve 1) and 1 μM Leu−TUPS (curve 2) in 5 mM Hepes/KOH (pH 7.0) excited at 372 nm.

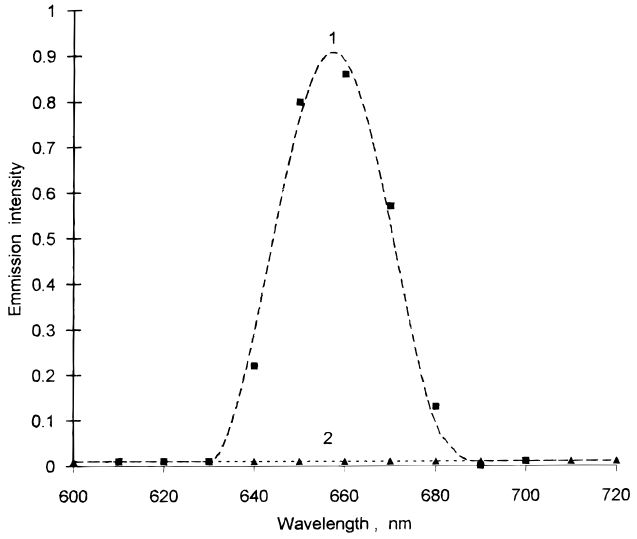

FIGURE 2: Phosphorescent emission spectrum of 10 μM Leu−TUPS in 5 mM Hepes/KOH (pH 7.0). Phosphorescence measurements were carried out in the presence of 10 μg/mL glucose oxidase, 1 μg/mL catalase, and 20 mM glucose (curve 1) and without oxygen-removing enzymes (curve 2). The photons emitted were counted during a 10 ms period, starting 0.1 ms after the excitation by a 372 nm flash.

form of the dye exhibits a phosphorescence with half-life time of ∼0.5 ms and a maximum at 658 nm (see Figure 2). The phosphorescence was extremely sensitive to dioxygen (an efficient triplet quencher); no emission was detected in

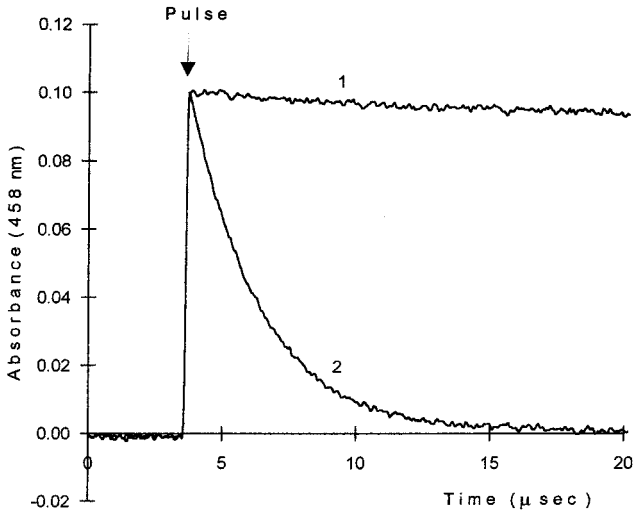

FIGURE 3: Absorbance transients of Leu−TUPS. 20 μM Leu−TUPS in 5 mM Hepes/KOH (pH 7.0), containing 20 mM glucose was irradiated by a 3 mJ pulse of a Nd:YAG third harmonic frequency ($\lambda = 355$ nm). The absorbance transients associated with formation and relaxation of TUPS excited state were measured by the 458 nm band of an argon laser in the absence (curve 1) and presence (curve 2) of oxygen as described in Materials and Methods. Each tracing is an average of 20 successive pulses.

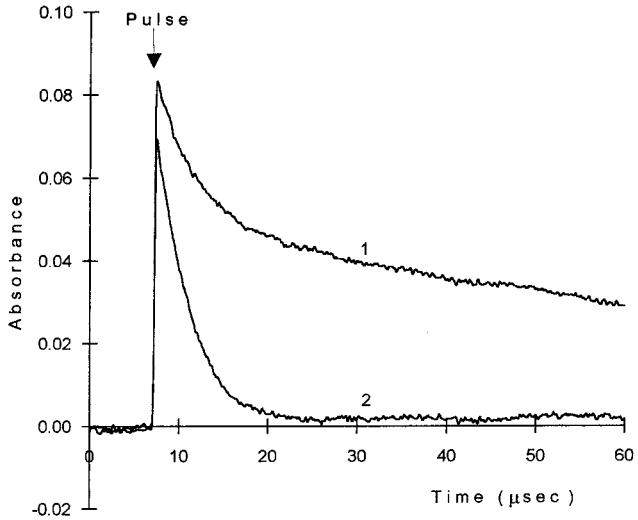

FIGURE 4: Absorbance transients of Leu−TUPS in the presence of RB. 20 μM Leu−TUPS, in a solution containing 5 mM Hepes/KOH (pH 7.0), 20 mM glucose, 10 μg/mL glucose oxidase, 1 μg/mL catalase, and 50 μM RB was irradiated by a 3 mJ pulse of a Nd:YAG third harmonic frequency ($\lambda = 355$ nm). The absorbance transients were measured by the 458 (curve 1) and 487 nm (curve 2) bands of an argon laser.

the presence of the gas (Figure 2, curve 2). The optical and luminescent properties of TUPS were independent of the nature of the amino acid conjugated to the dye.

Photoinduced Transient Absorption Kinetics. At high photon flux (60−100 MW/cm^2), the TUPS molecule undergoes photoxidation. The photochemistry of this process is identical to that of pyranine (8-hydroxypyrene-1,3,6-trisulfonate) and was described in our previous study (see ref 9). As the intensity of the photon flux decreases, the probability of the two-photon processes diminishes. At a photon flux of 10 MW/cm^2 and less the laser pulse yields only a one-photon excited state of the dye: TUPS*. TUPS* in contrast to the ground state dye absorbs at 458 nm, allowing the process of formation and relaxation of the excited state of the dye to be monitored at this wavelength. The initial unresolved rise in absorbance in Figure 3 corresponds to the formation of TUPS*. The subsequent decay is due to the relaxation of the excited molecule to the ground state. The relaxation rate is sensitive to dioxygen and is strongly accelerated in aerobic conditions (Figure 3, curve 2).

The slow relaxation dynamics of the excited dye in anaerobiosis can be greatly accelerated by the addition of oxidants. The rate of TUPS* relaxation increases up to a hundred times by submillimolar concentrations of ferricyanide, quinones, rhodamines, eosins, and viologens. The reduced forms of quinones and ferrocyanide did not effect the relaxation rate. The reactions of TUPS* with the low-potential dyes: eosin Y, erythrosin B, methyl viologen, benzyl viologen, and RB are extremely fast and proceed with the second-order rate constants of $(1-5) \times 10^9$ M^{-1} s^{-1}. The relaxation dynamics shown in Figure 4, depicts the kinetic measurements with RB as electron acceptor. Relaxation when followed at 458 nm (Figure 4, curve 1) is biphasic: the rate of the fast phase increases with increasing RB concentration. The rate of the slow phase is independent of RB concentration but increases in the presence of electron donors. This behavior is consistent with oxidation of the excited dye by RB, the reaction which yields reduced RB and oxidized TUPS. When followed at 478 nm the reaction results in complete and monophasic decay (Figure 4, curve 2) as the absorbance of the oxidized form at this wavelength is negligible. The reduction of RB resulted in a negative absorption transient at 553 nm, the absorbance maximum of the oxidized form (see Figure 5). Being parallel processes in the same reaction, reduction of RB and oxidation of TUPS* proceed with the same rate. The absolute values of absorbance changes at 553 and 487 nm, however, are different. The molar extinction coefficient of $\epsilon_{487} = 25\,000 \pm 3\,000$ M^{-1} cm^{-1} was calculated for TUPS* by relating the decrement of RB absorbance at 553 nm [$\epsilon = 108\,000$ M^{-1} cm^{-1} (*10*)] to the incremental absorbance at 487 nm. As seen in the figure, 5 MW laser pulse results in transformation of more than 15% of the dye to the excited form. To quantitate the quantum efficiency of this process the amount of TUPS* generated by a single pulse was related to the absorbed light energy. A yield as high as 0.3 was calculated.

The reduced RB and oxidized TUPS radicals generated by the pulse undergo redox recombination, the reaction which brings the system to its initial prepulsed state. Reoxidation of RB is much slower than its reduction and takes place in hundreds of microseconds (Figure 5B). The redox cycle initiated by photoexcitation of TUPS can be repeated several hundreds of times without any decrease of signal amplitude. The dynamics of TUPS* oxidation at different concentrations of RB is depicted in Figure 6. The transients fit well with a single-exponential decay. The pseudo-first-order rate constant of TUPS* oxidation increases linearly with RB concentration from 50 to 150 μM as evident from the figure. The second-order rate constant of $(2.5 \pm 0.2) \times 10^9$ M^{-1} s^{-1}, calculated from the slope of the line in the insert to Figure 6, is compatible with that of diffusion-controlled reactions.

The crucial point for further application of TUPS as an intraprotein redox perturbant is the ability of the excited dye to reduce the cofactor groups of proteins. Time-resolved kinetics of photosensitized reduction of cytochrome *c* are shown in Figure 7. The decay of absorbance at 487 nm

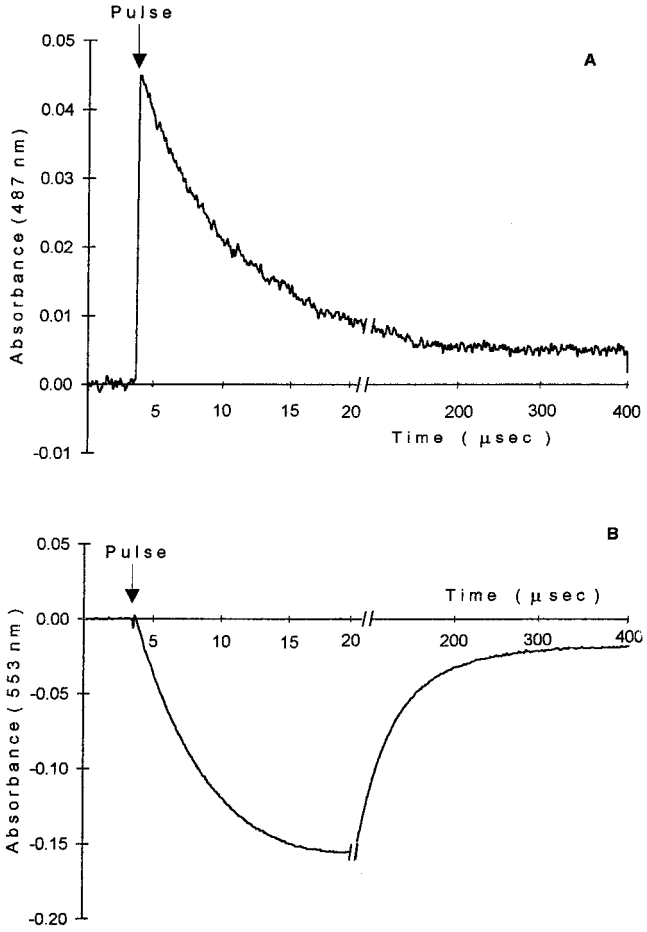

FIGURE 5: Transient kinetics for electron transfer in a solution containing 20 μM Leu−TUPS, 50 μM RB, 5 mM Hepes/KOH (pH 7.0), 20 mM glucose, 10 μg/mL glucose oxidase, 1 μg/mL catalase. The sample was irradiated by a 3 mJ pulse of a Nd:YAG third harmonic frequency (λ = 355 nm) in 1 mL (0.4 cm optical path) four-face quartz semi-microcuvette with front face of 1 cm and depth of 0.4 cm. The probing beam, either the 487 nm (A) band of a CW argon laser or the output of "Coherent" CR-599 dye laser with rhodamine-110 dye at 553 nm (B), was crossing the 0.4 cm side of cuvette.

corresponds with oxidation of the dye excited state by cytochrome *c*. Reduction of the later is seen as a monophasic increase of absorbance at 550−556 nm. The rate of the redox reaction increases linearly with cytochrome *c* concentration (not shown); the second-order rate constant for the heme reduction obtained from these data was $(3.4 \pm 0.3) \times 10^9$ M^{-1} s^{-1}, the value which is compatible with that of diffusion-controlled reactions. Reduction of the cytochrome's heme by the excited dye in the collisional complex may proceed as a single step reaction or may be mediated by reduced amino acid radicals, generated during the reaction of the protein with TUPS*. To investigate whether the interaction of the excited dye results in reduction of the protein's amino acids, the effect of free amino acids on TUPS* relaxation rate was checked. The relaxation rate was shown to be unaffected by a 10 mM concentration of any of the twenty standard protein amino acids.

DISCUSSION

Introduction of external photosensitive elements into the assay made it possible to use time resolved spectroscopy to elucidate electron transfer mechanisms in initially non-photosensitive redox systems. The experimental methods

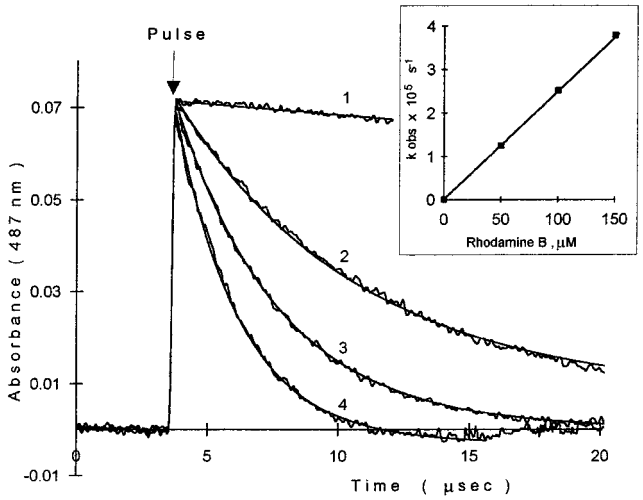

FIGURE 6: The dynamics of TUPS* oxidation by RB. 20 μM Leu−TUPS in 5 mM Hepes/KOH (pH 7.0) (curve 1), containing 50 (curve 2), 100 (curve 3), and 150 μM (curve 4) RB. The sample was purged with Ar and irradiated by a 3 mJ pulse of a Nd:YAG third harmonic frequency (λ = 355 nm) as described in Materials and Methods. The transients measured at 487 nm were fit by a single-exponential decay (solid line) with rate constants equal to $(1.8 \pm 0.1) \times 10^3$ s^{-1}; $(1.2 \pm 0.1) \times 10^5$ s^{-1}; $(2.5 \pm 0.1) \times 10^5$ s^{-1}; $(3.8 \pm 0.1) \times 10^5$ s^{-1}. The insert depicts the dependence of the calculated rate constants on the concentration of RB. The second-order rate constant calculated from the slope was $k = (2.5 \pm 0.2) \times 10^9$ M^{-1} s^{-1}.

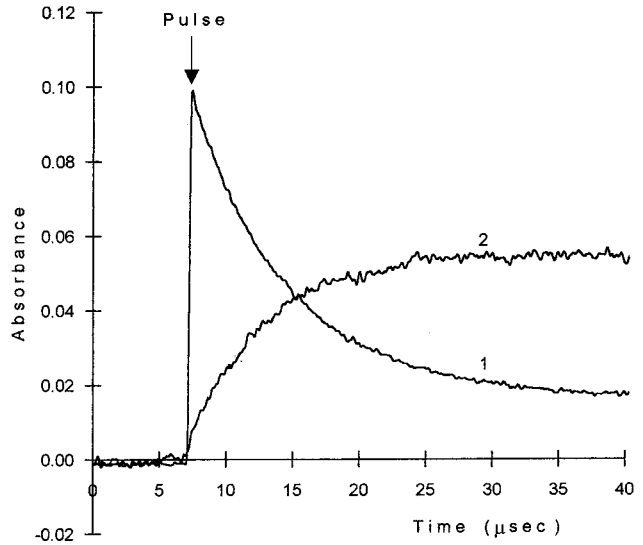

FIGURE 7: Transient kinetics for electron transfer in a solution containing 50 μM Leu−TUPS, 50 μM cytochrome *c*, 5 mM Hepes/KOH (pH 7.0), 100 mM KCl, 50 μM ferricyanide, 20 mM glucose, 10 μg/mL glucose oxidase, 1 μg/mL catalase. The sample was irradiated by a 3 mJ pulse of a Nd:YAG third harmonic frequency (λ = 355 nm). The transient at 487 nm (curve 1) was due to the formation and decay of TUPS*. The transient at 550 minus 556 nm (curve 2) accounted for reduction of the protein's heme group.

currently in use employ triplet states of photoexcited flavins and ruthenium complexes (*3*−*6*). The efficiency of photosensitive elements is determined by the quantum yield and life time of the excited state. The TUPS molecule serves both criteria: the triplet state of the dye has a quantum efficiency of approximately 30%, and the life time of the excited state is rather long ($\tau_{1/2} \sim 0.5$ ms). The excited dye molecule is a strong reductant capable of donating its electron to a variety of acceptors. The redox properties together with a high yield and long life time of the excited state make

TUPS useful for initiation and analysis of electron transfer reactions in chemical and biological systems.

The triplet nature of the excited state is an exceptional property of the thiouredo form of the pyrenetrisulfonic acid molecule. No long-lasting excited states were detected either for the isothiocyano form of the dye, or for pyranine (1-hydroxypyrene-3,6,8-trisulfonate) and its 1-oxymethyl derivative. Photoexcitation of the latter compounds by 355 nm photons yields the first excited singlet state (*9*), which rapidly relaxes to the ground state, a process accompanied by the emission of a photon. In contrast, relaxation of the thiouredo adduct proceeds predominantly via intersystem crossing, a process which yields a triplet. High quantum efficiency of intersystem crossing (approximately 30% of the absorbed photons generate the triplet) results in strong quenching of fluorescence and in phosphorescence of TUPS. The photon emitted during relaxation of the triplet to the ground state has an energy of 1.88 eV, a value corresponding to the wavelength of 658 nm. This value, exhibiting the energy difference between the triplet and the ground states, is equal to the difference of their oxidation potentials. The redox potential for one electron oxidation of pyranine, has been estimated in our previous work (*9*) to be +0.88 eV. Assuming that the redox potentials of pyrenetrisulfonates do not differ substantially, we can make an estimation of the redox potential of the triplet state of TUPS; the calculated value is equal to −1 eV. The capability of TUPS* to reduce a variety of low-potential (from −0.3 to −0.6 eV) electron acceptors, such as viologens, eosins, and rhodamines agrees well with the low potential of the TUPS*/TUPS couple. The reactions of the above mentioned dyes with TUPS* are quite fast. The rate constant for bimolecular oxidation of TUPS* by RB [$E_m = -0.54$ eV (*13*)] was estimated to be $(2.5 \pm 0.2) \times 10^9$ M^{-1} s^{-1}, a value compatible with that for the diffusion-controlled reactions. Apparently, the rate of oxidation is controlled by the encounter of the reactants, rather than by an energy barrier for electron transfer along the reactants coordinate within the encounter complex.

The goal of this study was the development of a photosensitive label for studies of electron transfer in proteins. In addition to its attractive electron transfer properties the dye molecule can be attached to lysine residues via its isothiocyano moiety. The capacity of photochemical generation of a potent reductant bound directly to proteins provides promising avenues for further research. Application of the approach for measurements of long-range electron transfer in cytochrome *c* is discussed in the accompanying paper (*14*).

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REFERENCES

1. Gunner, M. R., Robertson, D. E., and Dutton, L. P. (1986) *J. Phys. Chem. 90*, 3783−3795.
2. Moser, C. C., Keske, J. M., Warncke, K., Fariad, R. S., and Dutton, L. P. (1992) *Nature 355*, 796−802.
3. Ahmad, I., Cusanovich, M. A., and Tollin, G. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6724−6728.
4. Jung, J., and Tollin, G. (1981) *Biochemistry 20*, 5124−5131.
5. Winkler, J. R., Nocera, D. G., Bordignon, K. B., and Gray, H. B. (1982) *J. Am. Chem. Soc. 104*, 5798−5800.
6. Pan, L. P., Durham, B., Wolinska, J., and Millett, F. (1988) *Biochemistry 7*, 7180−7184.
7. Magner, E., and McLendon, G. (1989) *J. Phys. Chem. 93*, 7130−7134.
8. Zhou, J. S., and Kostic, N. M. (1991) *J. Am. Chem. Soc. 113*, 6067−6073.
9. Kotlyar, A. B., Borovok, N., Raviv, S., Zimanyi, L., and Gutman, M. (1996) *Photochem. Photobiol. 63*, 448−454.
10. Debruyne, I. (1983) *Anal. Biochem. 130*, 461−468.
11. Whitaker, J. E., Haugland, R. P., Moore, P. L., Hewitt, P. C., Reese, M., and Haugland, R. P. (1991) *Anal. Biochem. 198*, 119−130.
12. Molnar, E., Varga, S., Jona, I., and Martonisi, A. (1991) *Biochim. Biophys. Acta 1068*, 27−40.
13. Rao, P. S., and Hayon (1974) *J. Am. Chem. Soc. 96*, 1287−194.
14. Kotlyar, A., Borovok, N., and Hazani, M. (1997) *Biochemistry 36*, 15828−15833.

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