J|A|C|S
COMMUNICATIONS

Published on Web 09/29/2004

Photochemical Production of Nitric Oxide via Two-Photon Excitation with NIR Light

Stephen Wecksler, Alexander Mikhailovsky, and Peter C. Ford*

Department of Chemistry and Biochemistry, University of California, Santa Barbara, Santa Barbara, California 93106-9510

Received July 16, 2004; E-mail: ford@chem.ucsb.edu

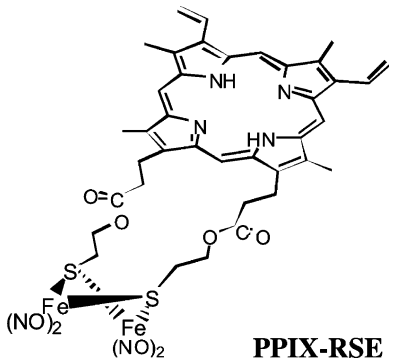
Nitric oxide (aka nitrogen monoxide) is involved in a broad array of biological functions including vasodilatation, immune response, and neurotransmission.[1] It is also a radiation sensitizer that enhances cell death in cell-cultures exposed to γ-radiation.[2] Thus, there is a continuing interest in targeted NO delivery in conjunction with γ-radiation treatment of tumors,[3] and this laboratory has been concerned with developing strategies to accomplish this task using photochemistry to trigger NO release from thermally stable precursors.[4−7] It would be particularly desirable to utilize near-infrared (NIR) wavelengths for in vivo photochemical activation owing to the 800−1100 nm spectral window of greatest light penetration in mammalian tissue.[8] In this context, we describe the successful photochemical generation of NO by two-photon excitation (TPE) of such a precursor by the use of NIR light.

Among compounds we have prepared for photochemical study are Fe/S/NO clusters known as the Roussin's salts and esters.[6,7] For example, we have demonstrated that 366−405 nm photolysis of Roussin's red salt ($Na_2[Fe_2(\mu\text{-}S)_2(NO)_4]$, RRS) releases NO with moderate quantum yields ($\Phi_{NO} = 0.02-0.13$ depending on conditions) and that, upon photolysis, these systems can sensitize cell killing by γ-radiation.[6a] However, RRS does not absorb strongly at the longer wavelengths desirable for an *in vivo* photochemical NO generator.

Accordingly, we have prepared red salt ester (RSE) derivatives $Fe_2(\mu\text{-}SR)_2(NO)_4$ that have pendant chromophores with the goal of modifying biological specificity and light-gathering properties by varying the R group. For example, the supramolecular complex PPIX−RSE ({μ-*S*,μ-*S*′-protoporphyrin-IX−bis(2-thioethyl)diester]-tetranitrosyl-diiron) which has a pendant protoporphyrin IX chromophore is photoactive under 546 nm continuous irradiation, i.e., under single-photon excitation (SPE).[7b] The pendant PPIX thus serves as a light-gathering antenna that is quenched by energy transfer to the $Fe_2S_2(NO)_4$ cluster with concomitant photochemical release of NO.[9] However, while this system demonstrates that pendant chromophores can serve as antennas for intramolecular sensitization of cluster photochemistry, the lower-energy cluster-centered states thus populated have relatively small reactivity, and the overall efficiency of the photochemical process is small. Moreover, 546 nm does not correspond to a light transmission window in tissue.

It is also known that the PPIX chromophore has a moderate TPE cross section ($\delta_{790\ nm} = 2$ GM).[10] In recognition of this feature, the present study describes the unprecedented photochemical reaction leading to NO release resulting from TPE of PPIX−RSE under the influence of 810 nm fs pulsed light.

TPE of free PPIX has been shown to generate a higher energy state (upconversion) with the same fluorescent spectrum as seen from higher energy excitation.[10] Thus, similar photochemistry should be observed for TPE as for SPE at about half the wavelength, regardless of the mode of excitation. In this context, we have carried



PPIX-RSE

out TPE experiments with PPIX−RSE parallel to the previously reported SPE studies of this species[7b] to explore the feasibility of photochemical generation of NO via TPE.

In these experiments solutions of free PPIX (13.5 μM), PPIX dimethyl ester (DME-PPIX) (11.6 μM), and PPIX−RSE (13.3 μM) were prepared such that absorbances at the Soret band ($\lambda_{max} \sim 406$ nm) were nearly identical (~2.0). (Supporting Information Figure S-1). TPE fluorescence measurements were performed using a mode-locked Ti:Sapphire laser (SpectraPhysics Tsunami) generating ~100 fs pulses with central wavelength 810 nm and repetition rate 80 MHz.[14] The solutions were irradiated for ~3-min periods while the TPE induced fluorescence was monitored over the range 550−750 nm. Although the absorption spectra were nearly the same, the intensities of the TPE-induced fluorescence differed markedly, the intensity from PPIX−RSE being ~4% that from the other two (Figure 1). These observations are consistent with the behavior of PPIX−RSE observed under 404 nm continuous excitation.[7b] The fluorescence from PPIX−RSE was substantially quenched relative to that of PPIX and DME-PPIX, consistent with relatively efficient energy transfer from the porphyrin singlet excited state of the antenna to the iron sulfur cluster.

In parallel experiments, NO was shown to be generated via TPE of PPIX−RSE solutions by using a nitric oxide electrochemical sensor (Amino-700 from Innovative Instruments). Solutions of PPIX−RSE (4.1 μM) in distilled, aerated THF were irradiated with the NIR laser under conditions identical to those for TPE fluorescence measurements. Exposure to the NIR laser varied from 1 to 3 min. Aliquots (50 μL) were withdrawn from the irradiated solutions and injected into a beaker containing 10 mL of deionized water in which the sensor was immersed. (The diluted PPIX−RSE concentration would then be ~20 nM.). Upon injection of the photolysis solution, immediate signal increases indicating NO generation were seen (Figure 2). The first three injections were of PPIX−RSE solutions that had irradiated at 810 nm for 1, 1, and 3 min, respectively, and the last two were from PPIX−RSE solutions that had not been irradiated (to evaluate the NO produced from

10.1021/ja045710+ CCC: $27.50

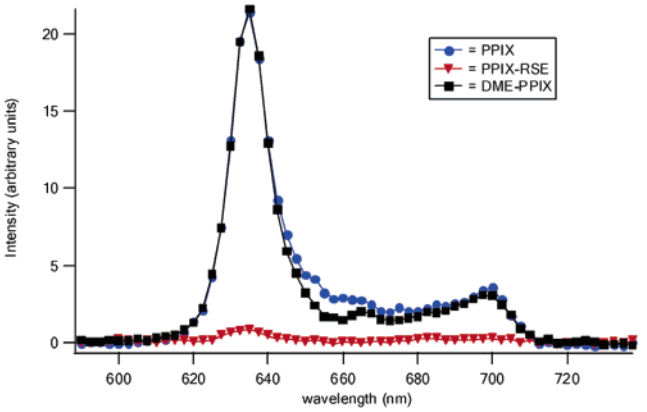


Figure 1. Fluorescence spectra (550−750 nm) of PPIX (13.5 μM), DME−PPIX (11.6 μM), and PPIX−RSE (13.3 μM) in THF solution, resulting from TPE excitation of solutions pumped with 100 fs pulses at 810 nm.

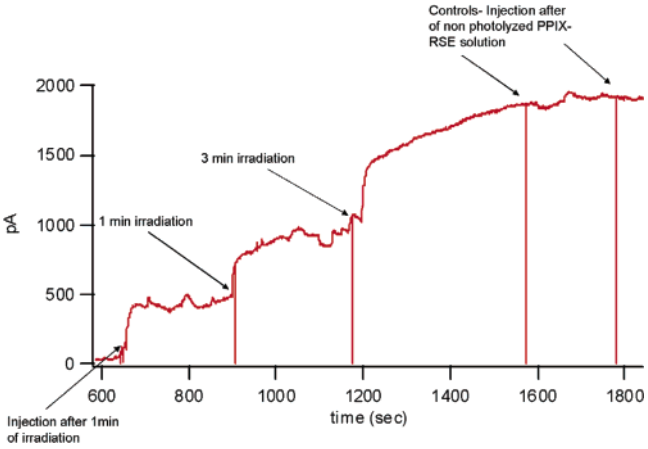


Figure 2. NO electrode response to 50 μL injections of photolyzed PPIX−RSE solutions in distilled, aerated THF. Samples were irradiated with 100 fs laser pulses (80 MHz) at 810 nm for 1−3 min intervals. The electrode was calibrated from 50 to 400 nM NO solutions prepared from acidified nitrite in the presence of NaI.

thermal decomposition). Clearly, the solutions subjected to TPE excitation liberated NO, while the controls did not.

The concentrations of the free NO released can be estimated from calibration curves as 2, 3, and 5 nM, respectively, from the irradiation experiments and ~0 nM from the controls. It was previously demonstrated that under continuous excitation RSE compounds release all four NO's from the cluster.[7a] If one assumes this also to be true of PPIX−RSE under TPE, then the first minute of illumination led to ~2.5% photodecomposition of this material. However, these estimates should be qualified, given documented difficulties encountered in quantitative electrochemical measurements of NO.[6b]

Further evidence for net photoreaction was obtained via positive ion electrospray ionization mass spectroscopy. ESI+ MS spectra of the solutions were obtained before and after 810 nm irradiation with 100 fs pulses. There was little difference between the initial and thermal control solutions (the main peak before irradiation being *m*/*z* 913, PPIX−RSE + H^+), but upon TPE several new peaks become apparent. Although some *m*/*z* 913 remained, a large peak at *m*/*z* 441 also appears with the expected isotopic pattern for a doubly charged iron species. This would be consistent with the doubly charged ion $[PPIX-RSE-NO]^{2+}$.

In conclusion, we have demonstrated that NO can be photochemically generated via TPE with 810 nm light of a suitable precursor, in this case the supramolecular complex PPIX−RSE. This is also a rare example of direct photochemical generation of a molecular species by TPE.[11,12] The TPE fluorescence data indicate that efficient energy transfer occurs from the porphyrin chromophore to the $Fe_2S_2(NO)_4$ core, resulting in fluorescence quenching and NO labilization. The advantages of such TPE photochemistry for NO delivery to biological targets are severalfold. The most obvious is the NIR spectral window in which one can operate in mammalian tissue.[8] However, another is that two-photon absorption is proportional to the square of the incident radiation intensity; thus, excitation efficiency falls of rapidly from the focal point. As a consequence, much greater spatial selectivity of excitation can be achieved in comparison to SPE. These properties have generated considerable interest in compounds that respond to TPE for applications in photodynamic therapy[15] and in biological imaging.[16] Continuing studies in this laboratory are focused on tailoring the NO precursors such as the Roussin's esters into compounds with appropriate solubility and biological specificities and with chromophores having larger TPE cross sections.

Acknowledgment. These studies were supported by the National Science Foundation (CHE-0352650).

Supporting Information Available: Absorption spectra of Figure 1 solutions. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) *Nitric Oxide: Biology and Pathobiology*; Ignarro, L. J., Ed.; Academic Press: San Diego 2000. (b) *Nitric Oxide and Infection*; Fang, F. C., Ed.; Kluwer Academic/Plenum Publishers: New York, 1999. (c) Wink, D. A.; Hanbauer, I.; Grisham, M. B.; Laval, F.; Nims, R. W.; Laval, J.; Cook, J.; Pacelli, R.; Liebmann, J.; Krishna, M.; Ford, P. C.; Mitchell, J. B. *Curr. Top. Cell. Reg.* **1996**, *34*, 159.

(2) Howard-Flanders, P. *Nature (London)* **1957**, *180*, 1191.

(3) Mitchell, J. B.; Wink, D. A.; DeGraff, W.; Gamson, J.; Keefer, L. K.; Krishna, M. C. *Cancer Res.* **1993**, *53*, 5845.

(4) Ford, P. C.; Bourassa, J.; Miranda, K.; Lee, B.; Lorkovic, I.; Boggs, S.; Kudo, S.; Laverman, L. *Coord. Chem. Rev.* **1998**, *171*, 185−202.

(5) (a) De Leo, M.; Ford, P. C. *J. Am. Chem. Soc.* **1999,** *121*, 1980−1981. (b) Works, C. F.; Ford, P. C. *J. Am. Chem. Soc.* **2000**, *122*, 7592−7593.

(6) (a) Bourassa, J.; DeGraff, W.; Kudo, S.; Wink, D. A.; Mitchell, J. B.; Ford, P. C. *J. Am. Chem. Soc.* **1997**, *119*, 2853−2860. (b) Kudo, S.; Bourassa, J. L.; Boggs, S. E.; Sato, Y.; Ford, P. C. *Anal. Biochem.* **1997**, *247*, 193−202. (c) Bourassa, J. L.; Ford, P. C. *Coord. Chem. Rev.* **2000**, *200*−*202*, 887−900.

(7) (a) Conrado, C.; Bourassa, J.; Egler, C.; Wecksler, S.; Ford, P. C. *Inorg. Chem.* **2003**, *42*, 2288−2293. (b) Conrado, C.; Wecksler, S.; Egler, C.; Magde, D.; Ford, P. C. *Inorg. Chem.* **2004**, *43*, 19, 5543−5549.

(8) Master, B. R.; So, P. T.; Gratton, E. *Biophys. J.* **1997**, *72*, 2405−2412.

(9) Another attractive feature is that protoporphyrin IX has been shown to localize in certain tumor tissues: (a) Livingston, R.; Watson, W. F.; McArdle, J. *J. Am. Chem. Soc.* **1949**, *71*, 1542. (b) Gouterman, M. In *The Porphyrins*; Dolphin, D., Ed.; Academic Press: New York, New York, 1978; Vol. 3, Part A, p 39. (c) Moan, J.; Sommer, S. *Cancer Lett.* **1993**, *21*, 167−174.

(10) Goyan, R. L.; Cramb, D. T. *Photochem. Photobiol.* **2000**, *72*, 821−827.

(11) Zhou, W.; Kuebler, S. M.; Braun, K. L.; Yu, T.; Cammack, J. K.; Ober, C. K.; Perry, J. W.; Marder, S. R. *Science* **2002**, *296*, 1106−1109.

(12) (a) Kim, H.-C.; Kreiling, S.; Greiner, A.; Hampp, N. *Chem. Phys. Lett.* **2003**, *372*, 899−903. (b) Kreiling, S.; Kim, H.-C.; Meyer, M.; Hampp, N.; Greiner, A. *Polym. Mater. Sci. Eng.* **2004**, *90*, 684−685.

(13) Xu, C.; Webb, W. W. *J. Opt. Soc. Am. B* **1996**, *13*, 481−491.

(14) The beam was nearly collimated within the span of the sample cell with average diameter of 135 μm. The mean power of the laser was 500 mW in all experiments. TPE fluorescence was collected at the right angle from the excitation beam direction, dispersed by a monochromator, and detected by a photomultiplier tube operating in a photon counting mode.

(15) (a) Dougherty, T. J.; Grindley, G.; Flel, R. *J. Natl. Cancer Inst.* **1974**, *55*, 115. (b) Bhawalkar, J. D.; Kumar, N. D.; Zhao, C.-F.; Prasad, P. N. *J. Clin. Lasers Med. Surg.* **1997**, *15*, 201−204.

(16) (a) Denk, W.; Strickler, J. H.; Webb, W. W. *Science* **1990**, *248*, 73. (b) Cheng, P. C.; Pan, S. J.; Bhawalkar, J. D.; Swiatkiewicz, J.; Samarabandu, J. K.; Liou, W. S.; He, G. S.; Prasad, P. N. *Scanning* **1996**, *18*, 14.

JA045710+