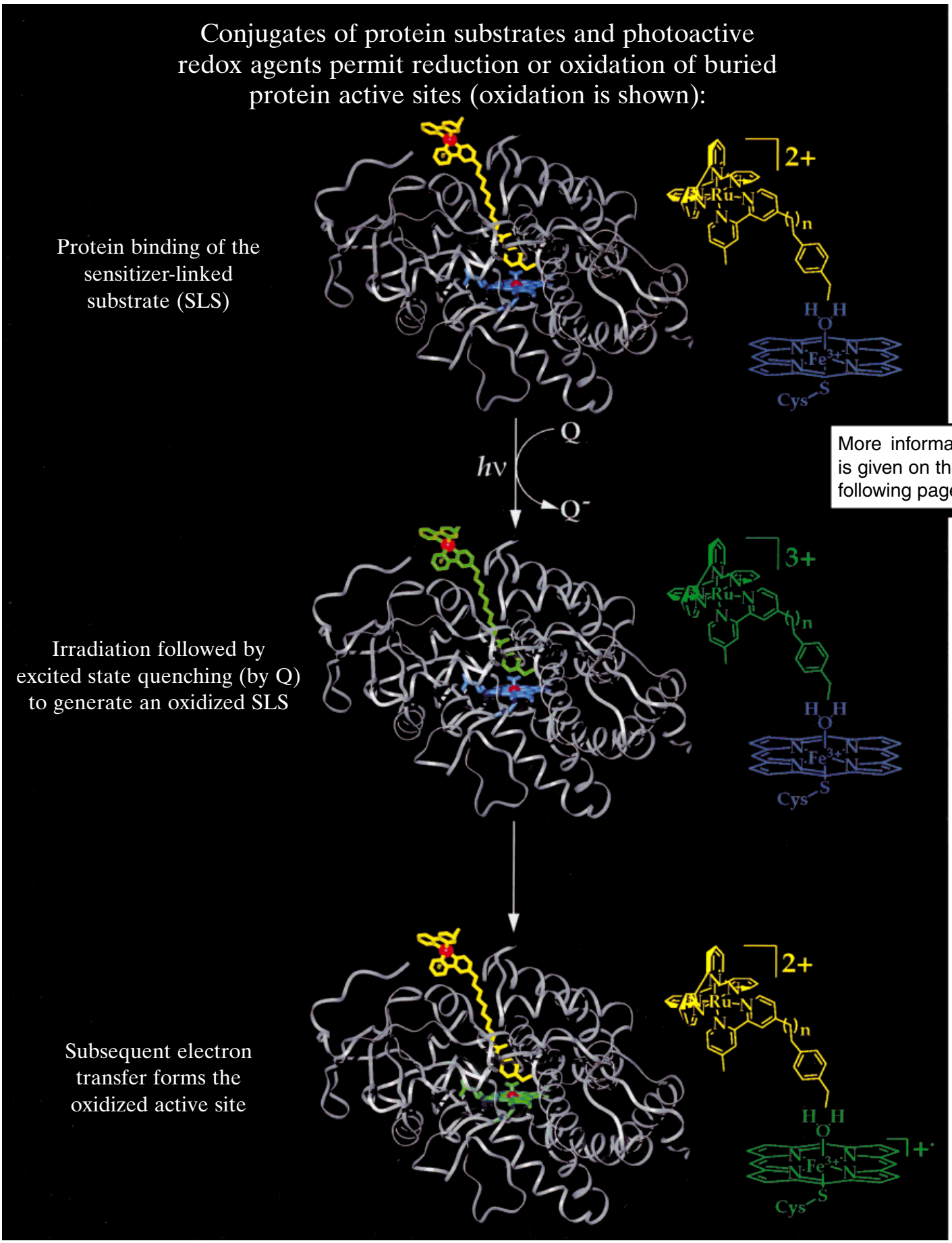
Conjugates of protein substrates and photoactive redox agents permit reduction or oxidation of buried protein active sites (oxidation is shown):



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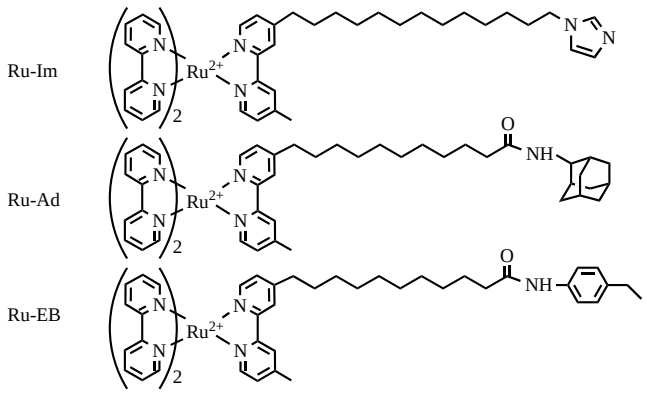
Substrates for Rapid Delivery of Electrons and Holes to Buried Active Sites in Proteins**

Jonathan J. Wilker, Ivan J. Dmochowski,
John H. Dawson, Jay R. Winkler,* and Harry B. Gray*

Rigorous characterization of metalloenzyme oxidation states is essential to understanding metabolic processes at a molecular level. Reactive intermediates in enzymatic catalysis are of special interest, but they are frequently too short-lived to be examined directly. A case in point is the high-valent heme that is believed to be a catalytic intermediate in the oxygenation reactions of cytochrome $P450_{cam}$ (P450);[1, 2] this oxidant has eluded detection thus far, raising questions concerning its role in the catalytic cycle.[1–3]

We have shown previously that reactive high-valent hemes of peroxidases can be prepared in solutions containing photogenerated ruthenium–diimine complexes as oxidizing agents.[4, 5] Our attempts to use the same approach with P450 have not met with success, however, owing in part to the inefficiency of electron transfer (ET) between external redox agents and a deeply buried heme. We have now developed a powerful photochemical method for the delivery of electrons and holes to buried redox sites. By tethering a Ru photosensitizer to a protein substrate, we have succeeded in reducing the P450 heme much more rapidly than has been possible previously, and we have also generated a hitherto unobserved oxidized state of the enzyme. The strategy of linking sensitizers to substrates opens the door to exploration of reactive redox states in enzyme interiors.

The photosensitizer $[Ru(bpy)_3]^{2+}$ (bpy = 2,2′-bipyridine) is linked through a hydrocarbon chain to a species with high affinity for the P450 heme pocket: imidazole (Im), adamantane (Ad), or ethylbenzene (EB).[6] Imidazole ligates the heme iron center directly,[7] whereas adamantane[8] and ethylbenzene[9] bind strongly to the hydrophobic cavity of the active site (Figure 1). Adamantanone displaces ligated water from the enzyme active site $[P_{Cys}Fe^{III}(OH_2)]$ (P_{Cys} is the protoporphyrin IX of P450 which is ligated through the thiolate group of a cysteine residue), to yield five-coordinate $[P_{Cys}Fe^{III}]$.[10] Binding of ethylbenzene, in contrast, leaves the six-coordinate resting state relatively unperturbed.



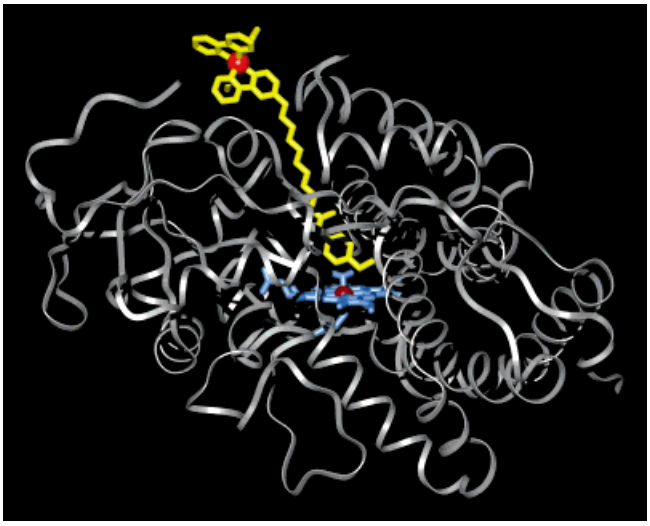


Figure 1. View of the (Ru-EB)–P450 complex with the Ru-EB alkyl chain placed in the channel to which the substrate has access (modeled from the crystal structure of P450: T. L. Poulos, *Methods Enzymol.* **1991**, *206*, 11–30). The hydrocarbon chain and protein substrate provide a well-coupled pathway for electron tunneling between the Ru and the heme. Energy minimization of (Ru-EB)–P450 was not performed.

Addition of stoichiometric amounts of Ru-Ad to ferric P450 shifts the Soret absorption maximum from 417 to 415 nm and creates a shoulder at 391 nm, indicating binding of the adamantyl moiety in the heme region.[11] This peak shift is attributed to lengthening of the Fe^{III}–OH_2 bond or partial displacement of water from the $[P_{Cys}Fe^{III}(OH_2)]$ resting state, both of which accompany the binding of adamantyl compounds in the heme cavity.[12] In addition to this absorbance change, there is a decrease in the lifetime of the excited state of Ru-Ad (Ru^{II*}-Ad).[13] The normally monophasic luminescence decay profile of Ru^{II*}-Ad ($k_1 = 2.2 \times 10^6\ s^{-1}$) becomes biphasic ($k_1 = 2.2 \times 10^6\ s^{-1}$, $k_2 = 7.7 \times 10^6\ s^{-1}$) in the presence of P450, with the faster phase accounting for 77% of Ru^{II*} decay. We attribute the rapid luminescence decay (k_2) to (Ru-Ad)–P450 interactions; the dissociation constant K_D is 0.69 μM for the complex formed between Ru-Ad and P450.[14]

The Soret absorption maximum of ferric P450 in the presence of equimolar amounts of Ru-Im shifts from 417 to 420 nm, indicating binding of the imidazole ligand to the heme iron center.[7] Luminescence decay of the (Ru^{II*}-Im)–P450 complex is biphasic ($k_1 = 2.2 \times 10^6\ s^{-1}$, $k_2 = 7.0 \times 10^6\ s^{-1}$).

[*] J. R. Winkler, H. B. Gray, J. J. Wilker, I. J. Dmochowski
Beckman Institute
California Institute of Technology
Pasadena, CA 91125 (USA)
Fax: (+1) 626-449-4159
E-mail: winklerj@cco.caltech.edu
hgcm@cco.caltech.edu

J. H. Dawson
Department of Chemistry and Biochemistry
University of South Carolina, Columbia, SC (USA).

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Approximately 68 % of Ru^{II*} decay occurs by the faster phase (k_2); $K_D = 1.5\,\mu M$ for (Ru-Im)–P450.

A 1:1 mixture of Ru-EB and P450 does not display an altered Soret absorption maximum; however, Ru^{II*}-EB is quenched in the presence of P450. The faster of two decay processes ($k_1 = 2.2 \times 10^6\ s^{-1}$, $k_2 = 1.2 \times 10^7\ s^{-1}$) accounts for 70 % of Ru^{II*}-EB decay, indicating $K_D = 1.0\,\mu M$ for the (Ru-EB)–P450 complex. Addition of excess camphor ($K_D \approx 1\,\mu M$ [2]) to (Ru-EB)–P450 displaces the Ru-linked substrate, as judged by an increased contribution of the slower luminescence decay process (k_1). We conclude that all three sensitizer-linked substrates bind tightly to the active site of P450.

Laser excitation of Ru^{II}-Im followed by reductive quenching with *para*-methoxy-*N,N*-dimethylaniline (*p*-MDMA)[15] yields a powerful reductant, Ru^{I}-Im ($E^0 = -1.24$ V versus the normal hydrogen electrode (NHE)).[16, 17] In the presence of P450, Ru^{I}-Im is converted rapidly into Ru^{II}-Im. Concomitant with this oxidation of Ru^{I} is reduction of the heme group, as evidenced by a shift of the Soret band from 420 to 445 nm.[7, 18] The rate constant k_{ET} for $Ru^{I} \rightarrow Fe^{III}$ electron transfer is $2 \times 10^4\ s^{-1}$ (Figure 2). Similar kinetics ($k_{ET} = 2 \times 10^4\ s^{-1}$) are observed upon reductive quenching of the

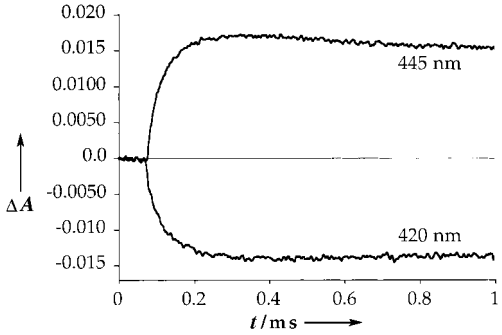

Figure 2. Plot of change in absorbance ΔA versus time t for the reaction of Ru^{I}-Im with P450 (10 μM ruthenium complex, 10 μM enzyme, 20 mM *p*-MDMA).

(Ru^{II*}-EB)–P450 complex; a shift of the Soret band from 417 to 390 nm results from the $Ru^{I} \rightarrow Fe^{III}$ reaction. Reductive quenching of the (Ru^{II*}-Ad)–P450 complex yields spectroscopic changes comparable to those seen for (Ru^{II*}-EB)–P450. This blue-shifted Soret band indicates that the reduction product is the previously unobserved $[P_{Cys}Fe^{II}(OH_2)]^-$ form of P450.[19] Further confirmation of heme reduction is the production of $[P_{Cys}Fe^{II}(CO)]^-$ ($\lambda_{max} = 448$ nm) upon steady-state visible irradiation of (Ru^{II}-EB)–P450 in the presence of *p*-MDMA and carbon monoxide. The relatively high rate of heme reduction in the photogenerated (Ru^{I}-EB)–P450 complex shows that a direct bond to the iron center is not required for efficient Ru–heme electronic coupling. Indeed, electron tunneling to the P450 active site through Ru-linked ethylbenzene is over two orders of magnitude faster than reduction by putidaredoxin ($k \approx 50\ s^{-1}$), a natural redox partner.[20]

The efficient coupling of the sensitizer-linked substrate to the heme can be exploited to generate a high-valent state of the enzyme. Oxidative quenching of the photoexcited species Ru^{II*}-EB by $[CoCl(NH_3)_5]^{2+}$ yields Ru^{III}-EB, a strong oxidant ($E^0 = 1.26$ V vs. NHE).[4, 17] The (Ru^{III}-EB)–P450 complex undergoes rapid heme-to-Ru^{III} electron transfer ($k_{ET} = 6 \times 10^3\ s^{-1}$), yielding an oxidized product with a Soret peak at 390 nm (Figure 3). This absorption change is not

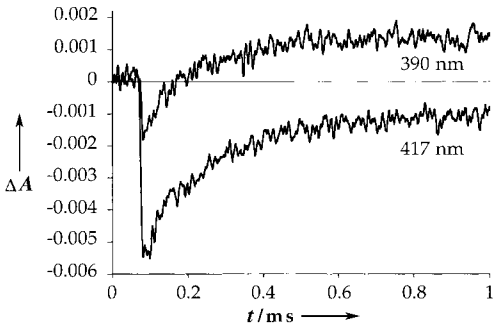

Figure 3. Plot of change in absorbance ΔA versus time t for the reaction of Ru^{III}-EB with P450 (10 μM ruthenium complex, 10 μM enzyme, 5 mM $[CoCl(NH_3)_5]^{2+}$).

observed upon laser photolysis of the (Ru^{II}-EB)–P450 complex in the absence of $[CoCl(NH_3)_5]^{2+}$. The oxidized species could be the porphyrin π-cation radical $[P^{\bullet+}_{Cys}Fe^{III}(OH_2)]^+$ or the Fe^{IV} species $[P_{Cys}Fe^{IV}(OH_2)]^+$. The blue-shifted Soret band in the spectrum of the oxidized heme is in accordance with the formulation of a radical;[21] indeed, porphyrin π-cation radicals often display Soret bands that are blue-shifted with respect to those of resting hemes.[22–24]

By employing sensitizer-linked substrates, we have prepared new oxidized and reduced states of P450 (Figure 4).

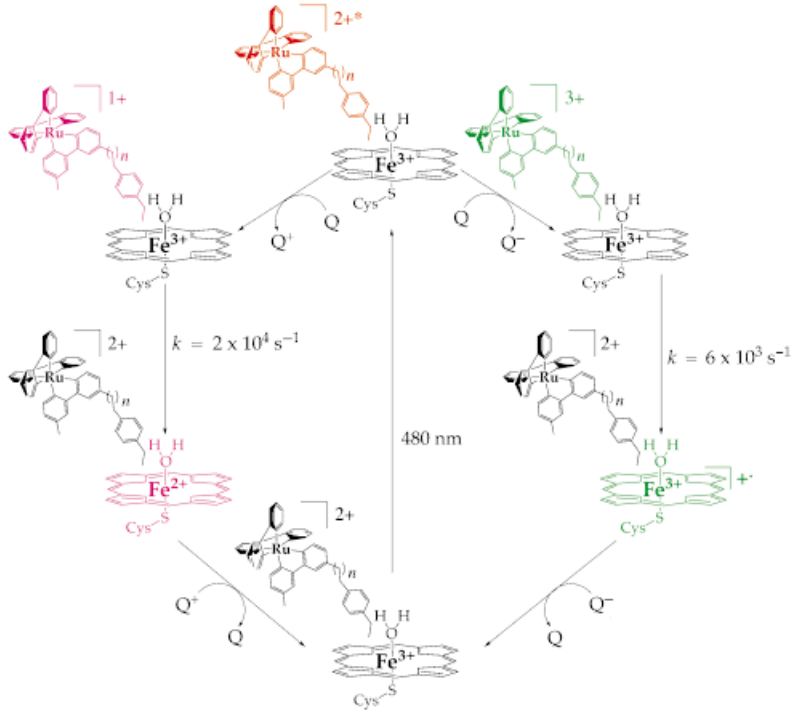

Figure 4. Schematic representation of the flash/quench reaction sequence for the preparation of new redox states in the (Ru-EB)–P450 complex. Q = quenching agent.

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These flash/quench methods provide a wide time window to study highly reactive forms of the enzyme. Both $[P_{Cys}Fe^{II}(OH_2)]^-$ and $[P^{+\bullet}_{Cys}Fe^{III}(OH_2)]^+$ are formed in about 0.1 ms and persist for approximately 100 ms. Improved design of sensitizers, quenchers, linkers, and substrates may lead to even faster electron and hole injection into P450 and other redox-active enzymes.

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Structural Investigation of a Dilithiated Phosphonate in the Solid State

Jürgen F. K. Müller,* Markus Neuburger, and Bernhard Spingler

The structural determination of reaction intermediates in organophosphonate anion chemistry has been stimulated by the extraordinary synthetic utility of these reagents.[1] A great body of information about the solid-state and solution structures of phosphorus-stabilized anions has been collected during recent years, and include lithiated phosphonates,[2] phosphinoxides,[3] phosphonamides, and thiophosphonamides.[4] Even though a more detailed picture of the characteristics was achieved, and important conclusions about the related reactivity were drawn, there some open questions still remain. For example, a general problem in the reactions of such monolithiated reagents with electrophiles occurs when more acidic products are formed. This usually leads to the formation of unwanted side products. To overcome this limitation, an attractive strategy that takes advantage of a dianionic intermediate has been developed.[5] Dicarbanions have already been used in several cases as supernucleophiles, and even in asymmetric reactions.[6] Despite the synthetic value of these reagents, there is still a demand for more information about the structures.[6l, 7] Our interest in the study of such dilithiophosphonates is an extension of our work in the development and application of chiral substituted dianions.[6l] Here we present the first solid-state structure of a geminal dilithiated phosphonate.

[*] Dr. J. F. K. Müller, M. Neuburger, B. Spingler
Institut für Anorganische Chemie der Universität
Spitalstrasse 51, CH-4056 Basel (Switzerland)
Fax: (+41) 61-267-10-18
E-mail: muellerju@ubaclu.unibas.ch

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