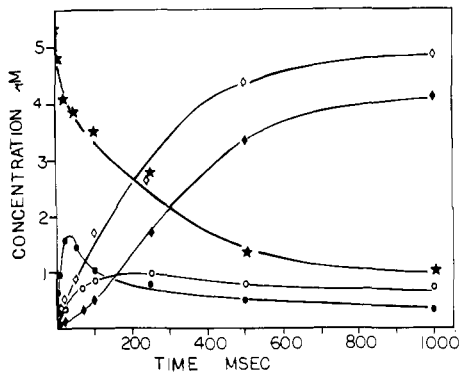


Figure 3. Time course for a single turnover of ATP, P_i, and pyruvate in the active site of Co^{2+}/NH_4^+-activated PPDK at 25 °C. The reaction mixture contained 6 μM [γ-^{32}P]ATP or [β-^{32}P]ATP, 20 μM PPDK active sites (S.A. ~ 15 μmol/min mg), 2.5 mM $CoCl_2$, 10 mM NH_4Cl, 2 mM P_i, 1 mM pyruvate, and 50 mM K^+Hepes (pH 7.0): (●) EPP, (○) EP, (★) ATP, (◇) PP_i, and (♦) PEP.

enzyme (40 μL) in a rapid quench apparatus.[10] The time courses for the reaction of Mg^{2+}/NH_4^+ activated PPDK with limiting [^{32}P]PEP in the absence and presence of the cosubstrates, AMP and PP_i, are depicted in Figure 1. Consistent with the uni-(PEP)uni(pyruvate) portion of the PPDK kinetic mechanism[5] we see that the phosphorylation of the enzyme by PEP occurs independent of PP_i and AMP. In a separate experiment (not shown) where the PEP was reacted with Mg^{2+}/NH_4^+-activated PPDK in a ratio of 1:20, the internal equilibrium constant (K_{eq}^{cat} = [EP·pyruvate]/[E·PEP]) was determined as 1.5.

The time course for the forward direction of the PPDK reaction was measured by using [γ-^{32}P]ATP to monitor EPP and PP_i formation and [β-^{32}P]ATP to monitor EPP + EP, PEP, and ADP formation. [^{14}C]ATP was used to demonstrate that radiolabeled nucleotide did not coprecipitate with the enzyme during the CCl_4 step of the reaction workup. The results obtained with the Mg^{2+}/NH_4^+-activated enzyme are shown in Figure 2. Both EPP and EP were formed as intermediates in the catalyzed reaction of ATP, P_i, and pyruvate, while ADP was not.

Because the level of EPP observed in the single turnover experiment was modest, conditions which would lead to enhanced accumulation of this intermediate were sought. In a separate study of PPDK catalysis the rate of isotope exchange from the P_β–O–P_α to the P_α=O position and from the P_β=O to P_γ–O–P_β position in [β-$^{18}O_2$,β,α-^{18}O]ATP was examined as a possible indicator of the relative rates of P_β–O–P_α and P_γ–O–P_β bond cleavage.[12] The $\beta \rightarrow \beta,\gamma$ and $\beta,\alpha \rightarrow \alpha$ positional isotope exchange (PIX) rates were equivalent for the Mg^{2+}/NH_4^+-activated enzyme, but, with the Co^{2+}/NH_4^+ activated enzyme, the $\beta,\alpha \rightarrow \alpha$ PIX occurred 2-fold faster than the $\beta \rightarrow \beta,\gamma$ PIX, and, with the Mn^{2+}/NH_4^+ enzyme, it was 30-fold faster. These findings suggested that with the Co^{2+}/NH_4^+- or Mn^{2+}/NH_4^+-activated enzyme the rate of EPP formation[13] may exceed the rate of its conversion to EP. Indeed, we found that the level of EPP which accumulates during a single turnover of ATP (6 μM) in the active site (18 μM) of the Mn^{2+}/NH_4^+-activated enzyme (EPP = 3.8 μM) is greater than that observed with the Co^{2+}/NH_4^+-activated enzyme (EPP = 1.7 μM) which in turn is greater than that observed with the Mg^{2+}/NH_4^+-activated enzyme (EPP = 0.3 μM).

The time course for ATP turnover in the active site of Co^{2+}/NH_4^+-activated PPDK is shown in Figure 3. These data clearly show that EPP is the first intermediate formed in this reaction. As the EPP is consumed the second intermediate, EP, and second product, PP_i, appear, and then after a short lag period the final product, PEP, appears. These observations demonstrate that the PPDK reaction proceeds by mechanism II of Scheme I and not by mechanism I.

Acknowledgment. This work was supported by a National Institutes of Health Grant GM-36260. We gratefully thank Kenneth Johnson for helpful discussions of this work and for use of his rapid quench apparatus in preliminary experiments.

(10) Reactions were quenched with 0.6 N HCl (164 μL); and the protein in the quenched sample was precipitated with CCl_4 (100 μL). The radiolabeled reactants and product(s) were separated by HPLC [Beckman Ultrasphere C18 analytical column, 25 mM K^+P_i, 2.5% triethylamine and 5% methanol (pH 6.5) isocratic elution]. The radioisotope content of the protein precipitate (dissolved in boiling 10 N H_2SO_4) and the HPLC fractions[5] was determined by using liquid scintillation techniques.

(11) Schendel, P. F.; Wells, R. D. *J. Biol. Chem.* **1973**, *248*, 8319.

(12) Pocalyko, D.; Mehl, A.; Dunaway-Mariano, D., unpublished.

(13) The uncertainty of the conclusion is due to the possibility that tortional equilibration at the α-P limits the P_β–O–$P_\alpha \rightarrow P_\alpha$=O PIX rate.

Doublet–Quartet Intersystem Crossing of Flavin Radical in DNA Photolyase

Tadashi Okamura

Laboratory of Physical Chemistry
Tokyo Zokei University
Hachioji, Tokyo 193, Japan

Aziz Sancar*

Department of Biochemistry, University of North Carolina
Chapel Hill, North Carolina 27599

Paul F. Heelis

Research Division
North East Wales Institute
Clwyd CH5 4BR, United Kingdom

Yoshinori Hirata and Noboru Mataga

Department of Chemistry, Faculty of Engineering Science, Osaka University
Toyonaka, Osaka 560, Japan

Received April 6, 1989

DNA photolyases repair UV (200–300 nm)-induced pyrimidine dimers in DNA in a light (300–500 nm)-dependent reaction.[1] The enzyme isolated from *Escherichia coli* has two chromophores, a stable neutral flavo-semiquinone radical (5-hydro-FAD, $FADH^0$)[2] and 5,10-methenyltetrahydrofolate.[3] Nanosecond flash photolysis studies[4,5] on the enzyme revealed a transient absorption band which decays with a rate constant of 0.8×10^6 s^{-1}. This absorption was assigned to an excited state of $FADH^0$, which decays by abstracting a hydrogen atom from a tryptophan residue[6] of the apoenzyme. This means that the radical must have an excited state with an intrinsic lifetime more than 1 μs. Even though this transient absorption was tentatively assigned to the lowest doublet state (D_1) of the flavin radical[5] we had reasons to believe that it may actually be the lowest quartet (Q_1) of $FADH^0$.[5] Since quartet states have not previously been identified in organic free radicals, we undertook a more detailed analysis of the excited states of the enzyme-bound $FADH^0$. In this study we have carried out picosecond laser photolysis on *E. coli* DNA photolyase in order to understand the dynamics of the photophysical processes in more detail. Our results indicate that the excited state species with 1 μs lifetime is the quartet of flavin radical.

E. coli DNA photolyase was prepared as described previously.[7] Enzyme concentration was 1.4×10^{-4} M with respect to the flavin radical. The enzyme was in a buffer containing 5×10^{-2} M Tris HCl, pH 7.5, 5×10^{-2} M NaCl, 10^{-3} M EDTA, 10^{-2} M dithiothreitol, and 50% glycerol.

(1) Sancar, A.; Sancar, G. B. *Annu. Rev. Biochem.* **1988**, *57*, 29.
(2) Jorns, M. S.; Sancar, G. B.; Sancar, A. *Biochemistry* **1984**, *23*, 2673.
(3) Johnson, J. L.; Hamm-Alvarez, S.; Payne, G.; Sancar, G. B.; Rajagopalan, K. V.; Sancar, A. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 2046–2050.
(4) Heelis, P. F.; Sancar, A. *Biochemistry* **1986**, *25*, 8163.
(5) Heelis, P. F.; Payne, G.; Sancar, A. *Biochemistry* **1987**, *26*, 4634.
(6) Heelis, P. F.; Sancar, A.; Okamura, T., manuscript in preparation.
(7) Sancar, A.; Sancar, G. B. *J. Mol. Biol.* **1984**, *172*, 223.

0002-7863/89/1511-5967$01.50/0

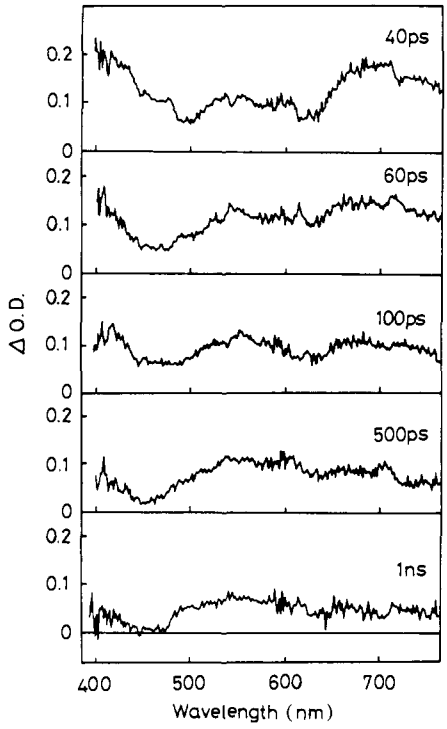


Figure 1. Transient absorption spectra of *escherichia coli* DNA photolyase with a photodecomposed second chromophore. Delay times from the exciting pulse are indicated in the figure.

Picosecond transient absorption spectra were measured at 293 K under aerobic conditions by using a microcomputer-controlled picosecond laser photolysis system with a mode-locked Nd^{3+}:YAG laser. Details of this system have been described elsewhere.[8] The third harmonic (355 nm, 1 mJ) with a 22 ps pulse width was used as an excitation source. Samples were contained in quartz cells of 1 cm optical path length, The absorption spectrum of the second chromophore,[2] 5,10-methenyltetrahydrofolate,[3] overlaps with that of the flavin radical. In order to investigate the excited states of $FADH^0$ independent of the second chromophore, the latter was selectively photodecomposed[5] to species that do not absorb at $\lambda > 300$ nm. The light source for photodecomposition was filtered light output ($\lambda > 360$ nm) of a high-pressure mercury lamp.

The transient absorption spectra of DNA photolyase containing $FADH^0$ but no second chromophore is shown in Figure 1. In these time dependent spectra, absorption bands which decay with a rate constant of ca. 10^{10} s^{-1} are clearly identified in the wavelength regions less than 500 nm and more than 600 nm. We assign these bands to the lowest excited doublet state (D_1) of the enzyme-bound $FADH^0$ which is formed by the very rapid internal conversion of the doublet manifold ($D_J \rightarrow D_1$) following laser excitation to the higher doublet state, D_J. This excited state (D_1, $\tau \simeq 10^{-10}$ s) would then decay nonradiatively to yield the long-lived ($\tau \simeq 1 \times 10^{-6}$ s) excited state which abstracts a hydrogen atom from the tryptophan residue. In addition to these rapidly decaying absorption bands, the spectra exhibit another band in the 300–500-nm region which decays very slowly. This band is probably due to the small amount of oxidized FAD contamination present in the photolyase sample. Similar transient absorption was reported for D-amino acid oxidase, a flavoenzyme whose cofactor is oxidized FAD.[9]

Photophysical processes of some organic free radicals, especially those of benzyl radicals, have been studied in detail.[10-15] These

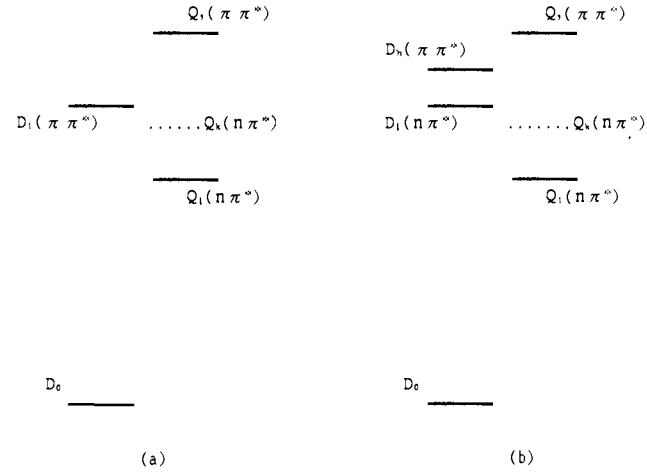


Figure 2. Possible energy level diagrams of $FADH^0$. (a) and (b) are diagrams with the D_1 state of $\pi\pi^*$ and that of $n\pi^*$, respectively.

studies were mainly concerned with the lifetimes of D_1 states. For example, the lifetime (τ_f) of the benzyl radical was found to be 1.28 μs at 77 K in EPA,[10] and those of its hydrocarbon derivatives were considerably shorter, in the range of 10^{-6}–10^{-7} s.[10,11] We believe the similarity between the fluorescence lifetime of benzyl radical and the lifetime of excited state of photolyase $FADH^0$ detected by nanosecond flash photolysis is coincidental as there is no similarity between the electronic spectra of the two radicals.[2,16] Anomalously long radiative lifetime of the benzyl radical is ascribed to the unique forbidden nature of the first electronic transition, $D_1 \leftarrow D_0$, which is not due to the selection rule and there is no a priori reason to think that the transition of $FADH^0$ would have the same character.

A reasonable approach to explain the photophysics of flavin radicals may be to consider two kinds of excited states of different spin multiplicities as in the case of usual stable organic molecules. Such an approach would require the consideration of quartet states. There is no direct experimental information available regarding excited quartet states of organic free radicals. A few theoretical studies[17-19] provide only very general outlines to the problem. The lack of phosphorescence and/or quartet–quartet absorption by hydrocarbon radicals may be taken as evidence that Q_1 ($\pi\pi^*$) states of these radicals lie above the D_1 ($\pi\pi^*$) states, and thus the Q_1 states cannot be populated enough to be detected. Such energetic order of the excited states is easily predicted by considering the major contributing electronic configurations for these $\pi\pi^*$ excited states. Theoretical calculations by some methods yield this same order.[16,17] However, for radicals such as flavin radicals which include heteroatom(s) within the conjugation system the situation may be rather different because of $n\pi^*$ excited states. For instance, the energy of the Q_1 ($n\pi^*$) state is expected to be lower than that of the D_1 ($\pi\pi^*$) state because it corresponds to a transition of an electron from a nonbonding orbital to the lowest empty orbital. Thus, for $FADH^0$, it seems reasonable to assume that the intersystem crossing to the $n\pi^*$ quartet state follows the internal conversion in doublet manifold. Intersystem crossing between $\pi\pi^*$ and $n\pi^*$ states is allowed by strong spin-orbit coupling (El-Sayed's rule) and, therefore, occurs very rapidly. This has been demonstrated for heterocyclic compounds by means of picosecond laser flash photolysis.[20-22] For example, the rate

(8) Mashuhara, M.; Ikeda, N.; Miyasaka, H. *J. Spectrosc. Soc. Jpn.* **1982**, *31*, 19.
(9) Karen, A.; Ikeda, N.; Mataga, N.; Tanaka, F. *Photochem. Photobiol.* **1983**, *37*, 495.
(10) Okamura, T.; Obi, K.; Tanaka, I. *Chem. Phys. Lett.* **1973**, *20*, 90; **1974**, *26*, 218.
(11) Okamura, T.; Tanaka, I. *J. Phys. Chem.* **1975**, *79*, 2728.
(12) Okamura, T.; Charlton, T. R.; Thrush, B. A. *Chem. Phys. Lett.* **1982**, *88*, 369.
(13) Mehnert, R.; Brede, O.; Helmstreit, W. *Z. Chem.* **1975**, *15*, 448.
(14) Topp, M. R. *Chem. Phys. Lett.* **1976**, *39*, 423.
(15) Obi, K.; Yamaguchi, H. *Chem. Phys. Lett.* **1978**, *54*, 448.
(16) Hiratsuka, H.; Okamura, T.; Tanaka, I.; Tanizaki, Y. *J. Phys. Chem.* **1980**, *84*, 285.
(17) Mori, Y. *Bull. Chem. Soc. Jpn.* **1961**, *34*, 1031; **1961**, *34*, 1035.
(18) Kruglyak, Y. A.; Mozdor, E. V. *Theor. Chim. Acta* **1969**, *15*, 374.
(19) Baudet, J.; Suard, M. *J. Chim. Phys.* **1970**, *67*, 1088.
(20) Hirata, Y.; Tanaka, I. *Chem. Phys. Lett.* **1976**, *41*, 336; **1977**, *25*, 381.
(21) Noe, L. J.; Degenkolb, E. O.; Rentzepis, P. M. *J. Chem. Phys.* **1978**, *68*, 4435.

constant for the buildup of the T_1 state of phenazine was measured to be 7×10^{10} s^{-1} in isooctane.[22] Fast decay of the transient absorption of $FADH^0$ in photolyase is probably due to the intersystem crossing of the same type (Figure 2): that from D_1 ($\pi\pi^*$) to Q_1 ($n\pi^*$) or to Q_k ($n\pi^*$) which lies between D_1 and Q_1. If D_1 is the $n\pi^*$ state, then we have to consider the intersystem crossing from D_1 ($n\pi^*$) to Q_1 ($n\pi^*$) or to Q_k ($n\pi^*$). In this case, vibronic coupling between D_1 ($n\pi^*$) and its neighboring D_n ($\pi\pi^*$) or between the lowest $\pi\pi^*$ quartet state Q_1 ($\pi\pi^*$) and $Q_i(n\pi^*)$ or $Q_k(n\pi^*)$ is necessary to cause the spin-orbit coupling. Fast intersystem crossing processes in the picosecond time region which presumably include vibronic couplings have been observed for aromatic ketones.[23-27]

In summary we conclude that intersystem crossing is a major relaxation process of the D_1 state of $FADH^0$ and that the excited states of lifetimes of ca. 100 ps and 1 μs we observe with nanosecond and picosecond flash photolysis, respectively, correspond to D_1 and Q_1 states of photolyase-bound flavin radical. Further experiments to prove this conclusion by transient spectroscopy of the flavin radical in solution and by quenching the doublet with heavy atoms are in progress.

(22) Hirata, Y.; Tanaka, I. *Chem. Phys. Lett.* **1976**, *43*, 568.
(23) Anderson, R. W., Jr.; Hochstrasser, R. M.; Lutz, H.; Scott, G. W. *J. Chem. Phys.* **1974**, *61*, 2500.
(24) Hochstrasser, R. M.; Lutz, H.; Scott, G. W. *Chem. Phys. Lett.* **1974**, *24*, 162.
(25) Kobayashi, T.; Nagakura, S. *Chem. Phys. Lett.* **1976**, *43*, 429.
(26) Scott, G. W.; Talley, L. D. *Chem. Phys. Lett.* **1957**, *53*, 431.
(27) Damschen, D. E.; Merritt, C. D.; Perry, D. L.; Scott, G. W.; Talley, L. D. *J. Phys. Chem.* **1978**, *82*, 2268.

π-Heterocyclic Complexes of Pentaammineosmium(II) and the Metal-Induced Cycloaddition of Pyrrole and Maleic Anhydride

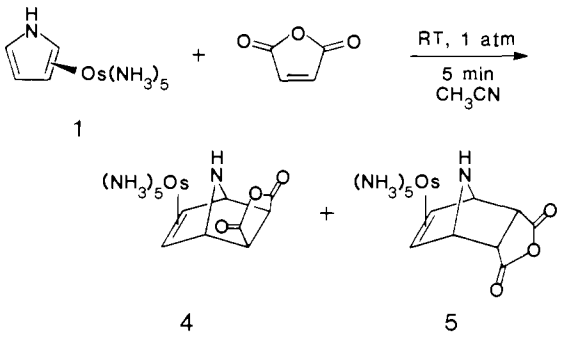
Rossella Cordone, W. Dean Harman, and Henry Taube*

Department of Chemistry, Stanford University
Stanford, California 94305
Received February 27, 1989

The isostructural heterocycles pyrrole, furan, and thiophene show markedly different reactivity toward cycloaddition to dienophiles.[1] Whereas furan reacts with maleic anhydride in a matter of seconds at room temperature,[1b] thiophene does so only at severe pressures,[1c] and the nitrogen analogue altogether fails to form cycloadducts without the addition of a catalyst.[1d] Judging from our previous experiences,[2] we anticipated that the intermediate $[(NH_3)_5Os]^{2+}$ would form stable π complexes with these heterocycles and, thus, we undertook an investigation of their reactivity toward cycloaddition.

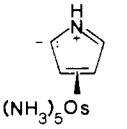
When $(NH_3)_5Os(OTf)_3$ is reduced over Mg^0 in the presence of an excess of the desired heterocycle, the complexes $[(NH_3)_5Os(2,3\text{-}\eta^2\text{-L})]^{2+}$ are obtained in high yield (L = pyrrole, **1**; furan, **2**; thiophene, **3**).[3] ^1H NMR spectra for these cations feature four inequivalent hydrocarbon resonances,[4] thereby ruling out η^1-coordination at the heteroatom. Contrary to what is observed for **2** or **3**, the pyrrole complex **1** displays resonances which are significantly broadened at room temperature; homonuclear decoupling at −25 °C reveals a dynamic process in which the metal tautomerizes between the 2,3- and 4,5-η^2-positions of the pyrrole ring.[5]

Scheme I. Cycloaddition Products for the Reaction of $[Os(NH_3)_5(pyrrole)]^{2+}$ and Maleic Anhydride



1 4 5

Chart I. The Azomethine Ylide Intermediate for the Complex $[Os(NH_3)_5(pyrrole)]^{2+}$



6

A ^1H NMR spectrum of an acetonitrile-d_3 solution containing the furan complex **2** (21 mM) and excess maleic anhydride (750 mM) shows no reactivity, even after 10 days. In an analogous experiment the thiophene complex **3** also fails to react over this time period. However, a rapid reaction occurs when **1** at the same concentration as above is exposed to 1 equiv of maleic anhydride in acetonitrile-d_3, as evidenced by the ^1H NMR spectrum of the mixture. After 5 min, the broad aromatic resonances originally observed for **1** have been completely replaced by three sharp singlets at 4.20 (2 H), 3.45 (2 H), and 3.14 (2 H) ppm (**4**) and another set of lines at 4.15 (dd, 2 H), 3.60 (dd, 2 H), and 3.23 (s, 2 H) ppm (**5**) in a 4:1 ratio.[6] The disappearance of the aromatic pyrrole resonances[7] and the observed NMR pattern point to the formation of two osmium(II) isomers containing 1:1 symmetrical cycloadducts of pyrrole and maleic anhydride.[8] When the reaction is performed with maleic anhydride-d_2, the resonances at 3.45 and 3.60 are absent; the high field peaks at 3.14 and 3.23 ppm agree well with the η^2-ethylene chemical shifts in $[(NH_3)_5Os(\eta^2\text{-}CH_2{=}{=}CH_2)]^{2+}$ (3.22 ppm) and can thus be assigned to the coordinated olefin protons of the adducts, and the remaining, low field resonances to bridgehead protons. From the splitting patterns it is concluded that **4** and **5** correspond to the *exo*- and *endo*-maleic anhydride isomers, respectively, with the metal moiety bound in the exo configuration for both species, as shown in Scheme I.

The cycloaddition reaction at −30 °C under the same conditions yields **4** and **5** in a ratio of 2:1; when the product solution is allowed

(1) (a) Acheson, R. M. *An Introduction to the Chemistry of Heterocyclic Compounds*; John Wiley and Sons: New York, 1976; Chapter III. (b) Lee, M. W.; Herndon, W. C. *J. Org. Chem.* **1978**, *43*, 518-19. (c) Kotsuki, H.; Nishisawa, H.; Kitagawa, S.; Ochi, M.; Yamasaki, N.; Matsuoka, K.; Tokoroyama, T. *Bull. Chem. Soc. Jpn.* **1979**, *52*, 544-46. (d) Matsumoto, K.; Hashimoto, S.; Ikemi, Y.; Otari, S. *Heterocycles* **1986**, *24*, 1835-39.
(2) Harman, W. D.; Taube, H. *J. Am. Chem. Soc.* **1987**, *109*, 1883. Cordone, R.; Taube, H. *J. Am. Chem. Soc.* **1987**, *109*, 8101.
(3) Synthesis of **1**: Pyrrole (1.5 mL) and Mg^0 (2 g) were added to a stirred suspension of $(NH_3)_5Os(OTf)_3$ (250 mg) and NaOTf (35 mg) in 5 mL of DME. Addition of CH_2Cl_2 (3 mL) to the filtered reaction mixture after 45 min resulted in a bright yellow precipitate which was either used without further purification (OTf salt) or purified by aqueous ion exchange chromatography and precipitated as a tetraphenylborate salt. E_p (acetonitrile, TBAH, ν = 200 mV/s) = −0.05 V (NHE). **1**: Calcd for $C_{52}H_{60}OsN_6B_2$: C, 63.67; H, 6.17; N, 8.57. Found: C, 63.44; H, 6.38; N, 8.40. Complexes **2** and **3** were prepared in similar manner by substituting DMA (0.5 mL) for NaOTf. **2**: $E_{p,a}$ = 0.67 V; Calcd for $C_{52}H_{59}OsO_1N_5B_2$: C, 63.61; H, 6.06; N, 7.13. Found: C, 63.37; H, 6.43; N, 6.89. **3**: $E_{p,a}$ = 0.45 V; Calcd for $C_6H_{19}OsS_3F_6O_6N_5$: C, 10.96; H, 2.91; N, 10.65; S, 14.63. Found: C, 11.09; H, 2.93; N, 10.57; S, 14.46.
(4) ^1H NMR in acetone-d_6 **1** (t = −25 °C): 7.47 (H_a, b, 1 H), 6.81 (H_b, d, 1 H), 6.65 (H_c, d, 1 H), 5.68 (H_d, t, 1 H), 5.36 (H_e, t, 1 H), 4.52 (b, 3 H); 3.32 (b, 12 H). **2** (20 °C): 7.50 (d, 1 H), 6.96 (d, 1 H), 6.18 (t, 1 H), 5.12 (t, 1 H), 4.76 (b, 3 H), 3.50 (b, 12 H). **3** (20 °C): 6.78 (d of d, 1 H), 6.75 (d of d), 6.05 (d of d, 1 H), 5.58 (d of d, 1 H), 4.90 (b, 3 H), 3.55 (b, 12 H).
(5) Partial spin saturation exchange was observed between H_b–H_c and H_d–H_e, an observation which indicates that the specific rate of tautomerization at −25 °C is on the order of seconds.
(6) Relative intensities were integrated against tetraphenylborate anion. *Cis*- and *trans*-ammine peaks at 3.05 (b, cis) and 3.80 (b, trans) are superimposed for 4 and **5**.
(7) No Michael addition products were detected in this reaction.
(8) A solid can be obtained by the addition of ether to the solution of the OTf^- salt. Analyses: Calcd for $C_8H_{22}OsS_2F_6O_9N_6$: C, 16.26; H, 3.00; N, 11.38. Found: C, 16.64; H, 3.21; N, 11.09.

0002-7863/89/1511-5969$01.50/0