Formation of Complexed Nitrosamines by Oxidation of Coordinated Ammonia in the Presence of Secondary Amines

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There is growing evidence that oxidation of reduced (−3) nitrogen species may play an important role in the biological formation of carcinogenic nitrosamines,[1,2] but the chemical literature offers little insight into the possible mechanisms involved. For example, immunostimulated macrophages can oxidize the −3 nitrogen in arginine to a nitrosating agent that converts secondary amines to their *N*-nitroso derivatives before production of free nitrite and nitrate can occur,[2] a reaction that appears to be without nonenzymatic precedent.

We now report that oxidation of coordinated ammonia at biologically reasonable potentials in the presence of secondary amines produces isolable complexes in which the nitrosamine ligand is bound to the metal center via the central (nitroso) nitrogen atom, a previously undescribed coordination mode for the nitrosamino function. The results suggest the hypothesis that the active site(s) of the enzyme(s) responsible for macrophage-induced and other biological nitrosamine formation may involve analogous metal centers capable of catalyzing both oxidation of reduced nitrogen species and subsequent nitrosamine-forming attack on ambient amines during a single turnover.

Electrochemical oxidation of the polypyridyl complex, $[(tpy)(bpy)Os^{II}(NH_3)]^{2+}$ (**1**),[3] dissolved in 0.5 M phosphate buffer in the presence of a 200-fold excess of diethylamine or morpholine at a measured pH of 6.8 and an applied potential of +0.65 V versus the sodium saturated calomel electrode (SSCE) proceeded as in eq 1. The electrochemical stoichiometry was established by

$$[(tpy)(bpy)Os^{II}(NH_3)]^{2+} + R_2NH \xrightarrow[-6H^+]{-6e^-,\ +H_2O} [(tpy)(bpy)Os^{II}(\overset{O}{\overset{\|}{N}}\text{-}NR_2)]^{2+} \quad (1)$$

1

bpy = 2,2'-bipyridine

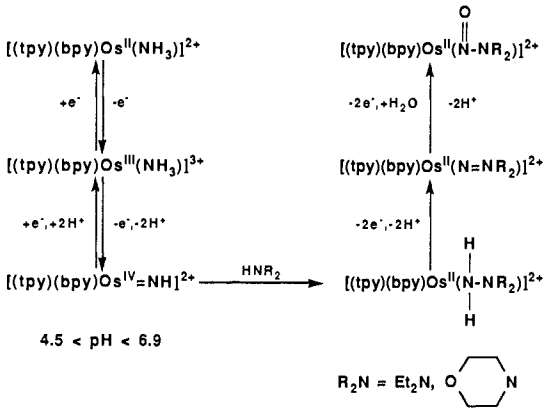
tpy = 2,2':6',2''-terpyridine

2 $NR_2 = NEt_2$

3 NR_2 = morpholino

coulometry ($n = 5.7 \pm 0.5$ electrons). The products[4] were isolated by extracting the aqueous solution with dichloromethane, concentrating the extracts, and precipitating the PF_6^- salts by addition to hexane.[5] The nitrosamine complexes have reversible Os(III/II) waves at $E_{1/2} = 0.56$ V (**2**) and 0.62 V (**3**) versus SSCE in acetonitrile containing 0.1 M tetraethylammonium perchlorate.

Scheme I. Proposed Mechanism for the Oxidative Conversion of **1** into **2** and **3**



Infrared (IR) and nuclear magnetic resonance (NMR) studies of the products support the structural assignment suggested in eq 1. Compounds **2** and **3** have ν_{NO} stretching frequencies at 1260 and 1220 cm^{-1}, respectively, compared to 1907 cm^{-1} for the nitrosyl complex, $[(tpy)(bpy)Os^{II}(NO)](PF_6)_3$ (**4**) (KBr pellet). For the ^{15}N-labeled analogues of **2** and **3**, prepared from $[(tpy)(bpy)Os^{II}(^{15}NH_3)]^{2+}$, ν_{NO} shifts from 1260 to 1248 cm^{-1} for **2** and from 1220 to 1206 cm^{-1} for **3**. The bands at 1260 and 1220 cm^{-1} are in the same energy region as the symmetric and asymmetric stretching modes of coordinated nitrite, $M-NO_2$,[6] and as ν_{NO} for other M–N(O)R complexes.[7,8] The IR comparisons suggest that the nitrosamine ligands are bound through the nitroso nitrogen.

^1H NMR spectra at 200 MHz of the nitrosamine complexes in acetonitrile-d_3 solution with tetramethylsilane as internal reference include sharp multiplets between δ 6.5 and 10 for the polypyridyl protons but broadened signals for the aliphatic protons. In the spectrum of the *N*-nitrosodiethylamine complex, **2**, a single broad methylene resonance is centered at δ 3.1 and a similarly broadened methyl peak appears at δ 0.8 at 20 °C. At −35 °C a pair of sharp methylene quartets (δ 2.70 and 3.75, $J = 7.4$ Hz) and two methyl triplets (δ 0.50 and 1.15) appear in the spectrum. The *N*-nitrosomorpholine complex, **3**, has four distinct but somewhat broadened CH_2 resonances at δ 3.85, 3.63, 3.10, and 2.60 at 20 °C. They sharpen to reveal fine structure at −35 °C. The spectra at the lower temperature indicate restricted rotation about the N–N bond as has been found for the free nitrosamines.[9] Rotational barriers for **2** and **3** were estimated to be 13.6 kcal/mol (**2**) and 15–16 kcal/mol (**3**) from their coalescence temperatures of 22 °C (**2**) and 58 °C (**3**).[10] The value for uncomplexed nitrosamines is approximately 23 kcal/mol[11] showing that this type of complexation reduces the barrier to rotation.[12]

(1) (a) Wagner, D. A.; Young, V. R.; Tannenbaum, S. R. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 4518–4521. (b) Saul, R. L.; Archer, M. C. *Carcinogenesis* **1984**, *5*, 77–81. (c) Wagner, D. A.; Shuker, D. E. G.; Bilmazes, C.; Obiedzinski, M.; Baker, I.; Young, V. R.; Tannenbaum, S. R. *Cancer Res.* **1985**, *45*, 6519–6522. (d) Stuehr, D. J.; Marletta, M. A. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 7738–7742. (e) Stuehr, D. J.; Marletta, M. A. *J. Immunol.* **1987**, *139*, 518–525. (f) Stuehr, D. J.; Marletta, M. A. *Cancer Res.* **1987**, *47*, 5590–5594.

(2) (a) Miwa, M.; Stuehr, D. J.; Marletta, M. A.; Wishnok, J. S.; Tannenbaum, S. R. *Carcinogenesis* **1987**, *8*, 955–958. (b) Iyengar, R.; Stuehr, D. J.; Marletta, M. A. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 6369–6373.

(3) Murphy, W. R., Jr.; Takeuchi, K.; Barley, M. H.; Meyer, T. J. *Inorg. Chem.* **1986**, *25*, 1041–1053.

(4) The fact that the reaction proceeds smoothly despite the nearly complete protonation of the basic amines at this pH is not without precedent. Classical, acid-catalyzed nitrosation of such secondary aliphatic amines by nitrite ion attains its maximal velocity at pH values much lower than this (Mirvish, S. S. *Toxicol. Appl. Pharmacol.* **1975**, *31*, 325–351).

(5) For the PF_6^- salt of **2**: Calcd for $C_{29}H_{29}N_7OsOP_2F_{12}$: C, 35.85; H, 3.01; N, 10.09. Found: C, 36.06; H, 2.98; N, 9.76. For the PF_6^- salt of **3**: Calcd for $C_{29}H_{27}N_7OsO_2P_2F_{12}$: C, 35.34; H, 2.76; N, 9.94. Found: C, 35.21; H, 3.05; N, 9.33.

(6) (a) Baran, E. J.; Müller, A. *Chem. Ber.* **1969**, *102*, 3915–3921. (b) Adeyemi, S. A.; Miller, F. J.; Meyer, T. J. *Inorg. Chem.* **1972**, *11*, 994–999. (c) Nakamoto, K. *Infrared Spectra of Inorganic and Coordination Compounds*; Wiley-Interscience: New York, 1982; pp 220–226.

(7) (a) Bowden, W. L.; Little, W. F.; Meyer, T. J. *J. Am. Chem. Soc.* **1974**, *96*, 5605–5607. (b) Bowden, W. L.; Little, W. F.; Meyer, T. J. *J. Am. Chem. Soc.* **1976**, *98*, 444–448.

(8) Nitrosyl stretching frequencies for free nitrosamines are much higher, e.g., ν_{NO} for *N*-nitrosodiethylamine is 1448 cm^{-1} (Fridman, A. L.; Mukhametshin, F. M.; Novikov, S. S. *Russ. Chem. Rev. (Engl. Transl.)* **1971**, *40*, 34–50).

(9) Karabatsos, G. J.; Taller, R. A. *J. Am. Chem. Soc.* **1964**, *86*, 4373–4378.

(10) The rotational barrier for **3** is given as a range until peak assignments can be confirmed. Calculations were performed as described in the following: Sohăr, P. *Nuclear Magnetic Resonance Spectroscopy*; CRC Press: Boca Raton, FL, 1983; Vol. 2, p 94.

(11) (a) Looney, C. E.; Phillips, W. D.; Reilly, E. L. *J. Am. Chem. Soc.* **1957**, *79*, 6136–6142. (b) Lunazzi, L.; Cerioni, G.; Ingold, K. U. *J. Am. Chem. Soc.* **1976**, *98*, 7484–7488.

(12) Facilitation of rotation about the N–N bond of an N–N–O system by interaction of the central nitrogen with an electrophilic center has also been reported recently by the following: Loechler, E. L.; Schneider, A. M.; Schwartz, D. B.; Hollocher, T. C. *J. Am. Chem. Soc.* **1987**, *109*, 3076–3087.

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The fact that this novel coordination mode lowers the N-N rotational barrier is understandable based on $d\pi(Os) \rightarrow \pi^*$ back donation to the N-bound nitrosamine ligand. Previously reported reactions between electrophiles (E^+), including metal ions, and nitrosamines have yielded predominantly O-bound products.[13] They have increased N-N rotational barriers relative to the uncomplexed nitrosamines.[14] Nitrosamines can also be protonated at the amino nitrogen.[14] The complexes reported here appear to be the first examples of nitrosamine complexes having the $[R_2N\text{-}N(E)=O]^+$ structure. We have been unable to obtain crystals suitable for X-ray diffraction.

A plausible mechanism for nitrosamine formation by eq 1 is shown in Scheme I. It is based upon results of earlier studies on the six-electron oxidation of **1** and its Ru analogue to the metal-nitro complexes.[3,15,16] The first step involves oxidation to Os(III), followed by loss of a second electron and two protons to give an osmium(IV)-imido intermediate. Before it can undergo further oxidation, the imido complex must be trapped by nucleophilic attack by the secondary amine to form an osmium(II)-hydrazine complex. A related reaction has been reported for the ruthenium analogue, which in aqueous solution undergoes nucleophilic attack by water to give the corresponding hydroxylamine complex (reaction 2).[15] At the potential of the elec-

$$[(tpy)(bpy)Ru^{IV}{=}NH]^{2+} + H_2O \longrightarrow [(tpy)(bpy)Ru^{II}(NH_2OH)]^{2+} \quad (2)$$

trolysis, the hydrazine complex, once formed, must be further oxidized, first to the diazenido complex and then by two more electrons coupled with water addition to form the nitrosamine product.

Both **2** and **3** form as products of an independent route which utilizes the electrophilic properties of the osmium nitrosyl complex, **4**, as a starting point (reaction 3).[17] When excess amine was

$$[(tpy)(bpy)Os^{II}NO]^{3+} + 2R_2NH \longrightarrow [(tpy)(bpy)Os^{II}(\overset{O}{\overset{\|}{N}}\text{-}NR_2)]^{2+} + R_2NH_2^+ \quad (3)$$

4

added to a suspension of **4** as its PF_6^- salt in dichloromethane, the solid dissolved, and the color of the solution changed from pale yellow to deep orange/brown. After a water wash and concentration of the dichloromethane solution, the product was precipitated by adding the dichloromethane solution to hexane. ^1H NMR analysis of the product dissolved in acetonitrile-d_3 revealed that it consisted of a small amount of bound nitrosamine complex (<10%) mixed with the corresponding nitro complex and other unidentified products. We have not yet found a satisfactory way to purify the nitrosamine products obtained by this route. Similar products were obtained when $[(tpy)(bpy)Ru^{II}(NO)]^{3+}$ was allowed to react with the secondary amines.

Warning! Most *N*-nitroso compounds are potent carcinogens[18] which must be handled, stored, and discarded with due respect for the possible hazards involved.

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(13) (a) Brown, R. D.; Coates, G. E. *J. Chem. Soc.* **1962**, 4723-4724. (b) Hünig, S.; Geldern, L.; Lücke, E. *Angew. Chem.* **1963**, *75*, 476. (c) Schmidpeter, A. *Chem. Ber.* **1963**, *96*, 3275-3279. (d) Schmidpeter, A. *Tetrahedron Lett.* **1963**, 1421-1424. (e) Klamann, D.; Koser, W. *Angew. Chem.* **1963**, *75*, 1104. (f) Hafner, K.; Wagner, K. *Angew. Chem.* **1963**, *75*, 1104-1105. (g) Kuhn, S. J.; McIntyre, J. S. *Can. J. Chem.* **1966**, *44*, 105-109. (h) Klement, U.; Schmidpeter, A. *Angew. Chem., Int. Ed. Engl.* **1968**, *7*, 470. (i) Klement, U. *Acta Crystallogr., Sect. B* **1969**, *25*, 2460-2465. (j) Axenrod, T. *Spectrosc. Lett.* **1970**, *3*, 263-265. (k) Büttner, G.; Cramer, J.; Geldern, L.; Hünig, S. *Chem. Ber.* **1971**, *104*, 1118-1135, and previous papers in the series. (l) Michejda, C. J.; Koepke, S. R. *J. Am. Chem. Soc.* **1978**, *100*, 1959-1960. (m) Koepke, S. R.; Kupper, R.; Michejda, C. J. *J. Org. Chem.* **1979**, *44*, 2718-2722. (n) Gouesnard, J. P.; Martin, G. J. *Org. Magn. Reson.* **1979**, *12*, 263-270. (o) Shustov, G. V.; Tavakalyan, N. B.; Shustova, L. L.; Chervin, I. I.; Kostyanovskii, R. G. *Bull. Acad. Sci. USSR (Engl. Transl.)* **1980**, *29*, 765-770. (p) Stöldt, E.; Kreher, R. *Chem. Ber.* **1983**, *116*, 819-822. (q) Keefer, L. K.; Hrabie, J. A.; Ohannesian, L.; Flippen-Anderson, J. L.; George, C. *J. Am. Chem. Soc.* **1988**, *110*, 3701-3702.

(14) Keefer, L. K.; Hrabie, J. A.; Hilton, B. D.; Wilbur, D. *J. Am. Chem. Soc.*, in press.

(15) Thompson, M. S.; Meyer, T. J. *J. Am. Chem. Soc.* **1981**, *103*, 5577-5579.

(16) Murphy, W. R., Jr.; Takeuchi, K.; Meyer, T. J. *J. Am. Chem. Soc.* **1982**, *104*, 5817-5819.

(17) Attempts to prepare osmium-nitrosamine complexes by direct reaction of a nitrosamine with other osmium derivatives have thus far proven unsuccessful.

(18) Preussmann, R.; Stewart, B. W. In *Chemical Carcinogens, Second Edition*; Searle, C. E., Ed.; ACS Monograph 182; American Chemical Society: Washington, DC, 1984; pp 643-828.

One-Dimensional Nuclear Overhauser Effect with Two-Dimensional Heteronuclear Multiple Quantum Coherence Detection: Proton-Proton Nitrogen-15 Correlation in T4 Lysozyme

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We have been working for some time on identification of as many proton and nitrogen resonances as reasonably possible in T4 lysozyme, with emphasis on the use of ^{15}N labeled samples and a variety of isotope-edited NMR techniques.[1] The X-ray structure of this 18.7 KD protein is already well known,[2] and our objective is to use these identifications for physical studies. Here we present one method which has allowed us to assign or help assign several resonances in the protein. The method is identical with one-dimensional nuclear Overhauser effect (NOE), except that a sensitive two-dimensional isotope-edited detection scheme is used which provides the extra resolution of a 2D experiment and the capability to edit spectra by selective isotope labeling.

One-dimensional NOE spectra are obtained as the difference between a control spectrum with off-resonance preirradiation that leaves all spins unperturbed and a spectrum for which the preirradiation frequency is coincident with a spin resonance. We extended this scheme to two-dimensional detection by replacing the 1D observe pulse with a version of proton-detected multiple quantum coherence detection[3a] which we call two dimensional forbidden echo or 2DFE.[1a] The NOE version is called saturation transfer 2DFE or ST2DFE.

The 2DFE map of uniformly ^{15}N enriched T4 lysozyme gives distinct peaks for many ^{15}NH amide and other groups (Figure 1a). Over 140 peaks have previously been classified by amino acid species by using selectively labeled samples,[1] and over 30 had been specifically identified by mutational substitution and by various edited NOE and double-label experiments.[1,3c] The ST2DFE experiment as applied here connects Cα proton frequencies with one or more of these amide peaks and is sometimes sufficient for identification of pairs of 2DFE amide peaks provided

(1) (a) Griffey, R. H.; Redfield, A. G.; Loomis, R. E.; Dahlquist, F. W. *Biochemistry* **1985**, *24*, 817-822. (b) Griffey, R. H.; Redfield, A. G.; McIntosh, L. P.; Oas, T. G.; Dahlquist, F. W. *J. Am. Chem. Soc.* **1986**, *108*, 6816-6817. (c) McIntosh, L. P.; Oas, T. G.; Dahlquist, F. W.; Griffey, R. H.; Redfield, A. G. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *84*, 1244-1248. (d) McIntosh, L. P.; Dahlquist, F. W.; Redfield, A. G. *J. Biomol. Struct. Dynam.* **1987**, *5*, 21-34.

(2) (a) Remington, S. J. et al. *J. Mol. Biol.* **1978**, *118*, 81-98. (b) Weaver, L. H.; Matthews, B. W. *J. Mol. Biol.* **1987**, *193*, 189.

(3) (a) Bax, A.; Griffey, R. H.; Hawkins, B. L. *J. Magn. Reson.* **1983**, *55*, 301-315. (b) Roy, S.; Redfield, A. G.; Papastavros, M. Z.; Sanchez, V. *Biochemistry* **1984**, *23*, 4395-4400. (c) Griffey, R. H.; Redfield, A. G. *Quart. Rev. Biophys.* **1987**, *19*, 51-82.

0002-7863/88/1510-6885$01.50/0