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Catalysis of NO Production by a Molybdoenzyme Model

Jill Murray, Donal Macartney, and Gregory R. J. Thatcher*

Department of Chemistry, Queen's University, Kingston, Ontario K7L 3N6, Canada

thatcher@chem.queensu.ca

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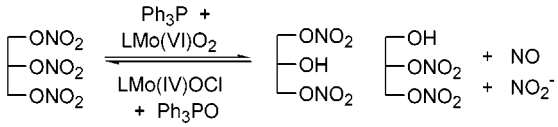
ABSTRACT



Nitric oxide (NO) is an important biological messenger molecule. Nitrates, including nitroglycerin (GTN), are clinically important vasodilators believed to be biotransformed in vivo to NO, a 3e$^-$ reduction. Molybdenum hydrotris-(3,5-dimethyl-1-pyrazolyl) borate complex (MoTPB) was shown to be an efficient catalyst of GTN degradation, with triphenylphosphine (Ph_3P) acting as reducing cofactor, producing significant amounts of NO. MoTPB/Ph_3P is an excellent enzyme model system, showing the feasibility of nitrate biotransformation mediated by a molybdoenzyme.

Nitric oxide (NO) is a biological messenger molecule produced in response to cell-specific external stimuli, with widespread and important biological roles, from vasodilation to neurotransmission.[1,2] Physiologically NO is produced from L-arginine by nitric oxide synthase (NOS) isozymes.[3] In addition to endogenous sources of NO, various exogenous NO donors have been reported. The nitrate ester nitroglycerin (GTN) has been in clinical use in treatment of angina for 125 years, and the biological activity of nitrates is certainly NO-mimetic.[4] It is generally believed that nitrate biological activity derives from biotransformation to NO in vivo by an undefined "mechanism-based" pathway and that saturation of this pathway leads to the clinical phenomenon of nitrate tolerance.[5,6] In contrast to mechanism-based biotransformation, nitrate degradation by "clearance-based" metabolism is believed to produce nitrite anion, NO_2^-, as product.[5] Reductive denitration may be achieved by a number of chemical routes.[7] Several proteins, including purified enzymes, have been shown to degrade GTN to glyceryl dinitrate (GDN) and NO_2^-, and evidence for NO release in complex biological systems does exist. However, no purified protein has been shown to catalyze nitrate reduction to yield significant amounts of NO.[5,6,8,9] Given the clinical importance of nitrates and research supporting potential, broader therapeutic applications, there is need to define pathways of mechanism-based biotransformation.[10]

The chemical conversion of a nitrate to NO is a 3e$^-$ reduction, requiring oxygen atom transfer (OAT). The molybdoenzyme, bacterial nitrate reductase, catalyzes reduction of NO_3^- to NO_2^-.[11] Therefore, mammalian molybdoenzymes, including aldehyde, sulfite, and xanthine oxidoreductases (XOR), which catalyze both OAT and e$^-$ transfer, are logical candidates for effecting nitrate biotransforma-

(1) Furchgott, R. F.; Zawadzki, J. V. *Nature* **1980**, *288*, 373. Murad, F. *Adv. Pharmacol.* **1994**, *26*, 19. Palmer, R. M. J.; Ignarro, L. J.; Buga, G. M.; Wood, K. S.; Byrns, R. E.; Chaudhuri, G. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 9265. Ferrige, A. G.; Moncada, S. *Nature* **1987**, *327*, 524. Moncada, S.; Higgs, E. A. *FASEB J.* **1995**, *9*, 1319.

(2) Butler, A. R.; Williams, D. L. H. *Chem. Soc. Rev.* **1993**, 233.

(3) Marletta, M. A. *Cell* **1994**, *78*, 927.

(4) Ignarro, L. J.; Buga, G. M.; Wood, K. S.; Byrns, R. E.; Chaudhuri, G. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 9265. Fung, H. L. *Eur. Heart J.* **1991**, *12*, 9.

(5) Bennett, B. M.; McDonald, B. J.; Nigam, R.; Simon, W. C. *Trends Pharmacol. Sci.* **1994**, *15*, 245.

(6) Thatcher, G. R. J.; Weldon, H. *Chem. Soc. Rev.* **1998**, *27*, 331.

(7) Swan, E. P.; Hayward, L. D. *Can. J. Chem.* **1956**, *34*, 856.

(8) NO has been observed from action of XOR on GTN, involving reductive denitration of GTN to NO_2^-, but "further reduction of NO_2^- [to NO] is very slow...": Doel, J. J.; Godber, B. L.; Eisenthal, R.; Harrison, R. *Biochim. Biophys. Acta* **2001**, *1527*, 81.

(9) In aerobic, buffered, aqueous solution, with cysteine (2 mM), GTN (1 mM) releases 25 nM NO after 10 min.: Artz, J. D.; Toader, V.; Zavorin, S. I.; Bennett, B. M.; Thatcher, G. R. J. *Biochemistry* **2001**, *40*, 9256.

(10) Smith, S.; Dringenberg, H. C.; Bennett, B. M.; Thatcher, G. R. J.; Reynolds, J. N. *NeuroReport* **2000**, *11*, 3883. al-Swayeh, O. A.; Clifford, R. H.; del Soldato, P.; Moore, P. K. *Br. J. Pharmacol.* **2000**, *129*, 343.

(11) Hille, R. *Essays Biochem.* **1999**, *34*, 125.

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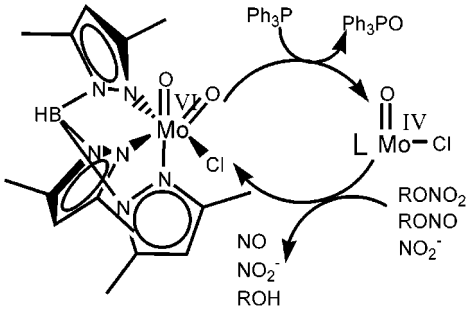
tion.[12] Biological arguments specifically implicating XOR have been made on the basis of the presence of XOR in the endothelium. Recently, Harrison has reported that the more facile 1e$^-$ reduction of both organic nitrites (RONO) and NO_2^- to NO is catalyzed by XOR.[13,14] However, the situation for nitrates, vis-à-vis XOR, is more complex: (1) Harrison demonstrated that anaerobic 2e$^-$ reduction of nitrates yields NO_2^- and occurs at the flavin, not at the Mo site of XOR,[8] and (2) Bennett provided data contraindicating a role for XOR in mechanism-based nitrate biotransformation.[15] The situation with XOR is further complicated, because (a) O_2^- is a product of XOR action that reacts rapidly with NO generating peroxynitrite,[16] and (b) NO has been reported to inhibit XOR.[13,17]

In situations in which biological systems or the enzymes themselves have too great a level of complexity to derive unambiguous conclusions, simple, nonprotein enzyme models provide a useful means of providing insight and predictions on enzyme structure and function.[18] Furthermore, enzyme models that successfully mimic enzyme function are biomimetics that are likely themselves to show interesting biological activity, a case in point being the superoxide dismutase mimics.[19] Given the complications surrounding the interactions of XOR with NO, NO_2^-, O_2^-, and nitrates, we have sought to develop a simple molybdoenzyme model system to ask the question: *Do molybdoenzymes, in general, possess the catalytic apparatus to carry out the 3e$^-$ reduction of nitrates to NO*?

The development of a model system requires a catalytically active Mo complex and a reducing cofactor, a task greatly assisted by the wealth of research on biomimetic Mo complexes.[20−22] Herein, we report the reduction of GTN to NO, by an oxidoreductase model system, consisting of MoTPB (Mo^{VI}-hydrotris-(3,5-dimethyl-1-pyrazolyl)-borate; $HB(Me_2pz)_3MoO_2Cl$)[20] with triphenylphosphine (Ph_3P) as the reducing cofactor. This represents the first report of a simple biomimetic system capable of rapid reduction of an organic nitrate and generation of a large flux of NO. The system is also truly catalytic and, further, is capable of reducing both NO_2^- and organic nitrite to NO (Scheme 1).

Scheme 1



Substrates included nitrates GTN and thioglyceryl dinitrate (TGDN; $HSCH_2CHONO_2CH_2ONO_2$);[23] *i*-amyl nitrite (IAN) and the activated nitrite phenoxyethyl nitrite (PEN); and NO_2^- (as the $Bu^n{}_4N^+$ salt in organic solvent and the Na^+ salt in aqueous solution). Control reactions were performed in the absence of each component of the model system. Although reactions are faster in dichloromethane solvent, it is not required for reaction, since other organic solvents may be used in its place. Further, reactions proceed both under aerobic (data presented herein) and anaerobic conditions. However, the requirement of a P(III) reductant for GTN reduction appears absolute as all other N- and S-centered reducing agents assayed did not increase reaction rates with MoTPB above controls.

Initial experiments used ^{31}P NMR to quantify MoTPB catalysis of oxygen transfer from GTN to phosphine. Phosphine oxidation was observed to be dependent on GTN concentration (Figure 1). Control reactions showed that the slow autoxidation of Ph_3P (20 mM) to Ph_3PO, in the absence of MoTPB, was accelerated by excess GTN (300 mM, $t_{1/2}$

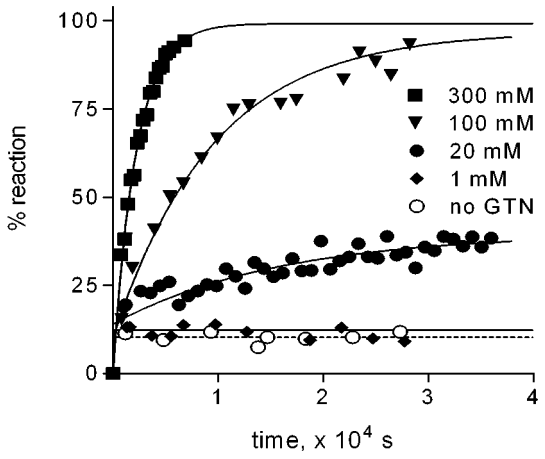


Figure 1. Percentage reaction of Ph_3P from relative integration, $Ph_3PO/(Ph_3P + Ph_3PO)$, of ^{31}P NMR signals (162 MHz, corrected for relaxation effects) obtained in aerobic CH_2Cl_2, at 25 °C, Ph_3P (20 mM), MoTPB (1mM), at various [GTN]. Pseudo-first-order curves fitted to include initial oxidation burst. Data points shown for one experiments are representative of triplicate sets.

(12) McMaster, J.; Enemark, J. H. *Curr. Opin. Chem. Biol.* **1998**, *2*, 201. Hille, R. *Chem. Rev.* **1996**, *96*, 2757.
(13) Doel, J. J.; Godber, B. L.; Goult, T. A.; Eisenthal, R.; Harrison, R. *Biochem. Biophys. Res. Commun.* **2000**, *270*, 880.
(14) Godber, B. L.; Doel, J. J.; Sapkota, G. P.; Blake, D. R.; Stevens, C. R.; Eisenthal, R.; Harrison, R. *J. Biol. Chem.* **2000**, *275*, 7757.
(15) Ratz, J. D.; McGuire, J. J.; Anderson, D. J.; Bennett, B. M. *J. Pharmacol. Exp. Ther.* **2000**, *293*, 569.
(16) Godber, B. L.; Doel, J. J.; Durgan, J.; Eisenthal, R.; Harrison, R. *FEBS Lett.* **2000**, *475*, 93.
(17) Ichimori, K.; Fukahori, M.; Nakazawa, H.; Okamoto, K.; Nishino, T. *J. Biol. Chem.* **1999**, *274*, 7763. Lee, C. I.; Liu, X.; Zweier, J. L. *J. Biol. Chem.* **2000**, *275*, 9369.
(18) Dong, S. D.; Breslow, R. *Chem. Rev.* **1998**, *98*, 1997.
(19) Spasojevic, I.; Batinic-Haberle, I.; Stevens, R. D.; Hambright, P.; Thorpe, A. N.; Grodkowski, J.; Neta, P.; Fridovich, I. *Inorg. Chem.* **2001**, *40*, 726. Batinic-Haberle, I.; Benov, L.; Spasojevic, I.; Fridovich, I. *J. Biol. Chem.* **1998**, *273*, 24521.
(20) Roberts, S. A.; Young, C. G.; Kipke, C. A.; Cleland, W. E., Jr.; Yamanouchi, K.; Carducci, M. D.; Enemark, J. H. *Inorg. Chem.* **1990**, *29*, 3650.
(21) Lim, B. S.; Holm, R. H. *J. Am. Chem. Soc.* **2001**, *123*, 1920.
(22) Smith, P. D.; Millar, A. J.; Young, C. G.; Ghosh, A.; Basu, P. *J. Am. Chem. Soc.* **2000**, *122*, 9298.

≈ 10 h), demonstrating for the first time a reaction between a P nucleophile and an organic nitrate functional group.[24] The further addition of a catalytic quantity of MoTPB (1 mM) led to complete phosphine oxidation within 2 h at 25 °C. At low or zero GTN concentration, a rapid stoichiometric oxidation of phosphine by MoTPB is observed (Figure 1). In addition, the rate of phosphine oxidation in the presence of excess GTN was observed to be dependent on MoTPB concentration.

The observed products of reductive degradation of GTN in vitro and in vivo are the GDN isomers and NO_2^-, which were also confirmed as products of MoTPB/Ph_3P reduction of GTN by HPLC/UV identification of GDNs and the Griess test for NO_2^-. However, in this system, reduction of GTN also generated a significant flux of NO, quantified by chemiluminescence detection of reaction headspace gas and dramatically illustrated by sequential addition of MoTPB and Ph_3P to CH_2Cl_2 solutions of nitrates (Figure 2). This contrasts

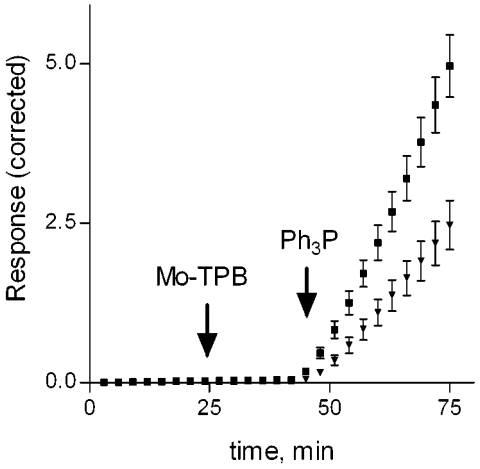


Figure 2. Response of NO-chemiluminescence detector after injections of MoTPB (0.9 mM) and Ph_3P (16.7 mM) to GTN (■, 1 mM) or TGDN (▼, 1 mM) in CH_2Cl_2 in septa-sealed, water-jacketed reaction vials, 25 °C. Headspace sampled by syringe injection (400 μL) directly into a chemiluminescence detector (Sievers Research Inc., Boulder, CO, model 207B). Data points are cumulative means (± s.e.) of sequential readings obtained in triplicate assays. A response of 2.0 corresponds to [NO] = 3 μM in headspace, by comparison with direct injection of standardized NO gas (Scott Air Products, PA).

observations in aqueous solution, where NO release from GTN is negligible, whereas TGDN spontaneously releases NO.[9,23] To further test this biomimetic system, DMSO (2.5 mM) was added as a competitive substrate leading to inhibition of GTN degradation and abolition of NO release from GTN (1 mM).

The simplest mechanisms for nitrate reduction involve initial 2e$^-$ reduction to either organic or inorganic nitrite, followed by subsequent 1e$^-$ reduction to NO:

$$RONO_2 + Ph_3P \rightarrow RONO + Ph_3PO \quad (1a)$$

$$RONO + 1e^- \rightarrow RO^- + NO \quad (1b)$$

$$RONO_2 + 2e^- \rightarrow RO^- + NO_2^- \quad (2a)$$

$$NO_2^- + Ph_3P \rightarrow Ph_3PO + NO + 1e^- \quad (2b)$$

As might be expected, the 1e$^-$ nitrite reductions are more facile than the 3e$^-$ reduction of GTN, with NO release being detected from both nitrites in the presence and absence of MoTPB (Figure 3). Organic nitrite and NO_2^- are therefore

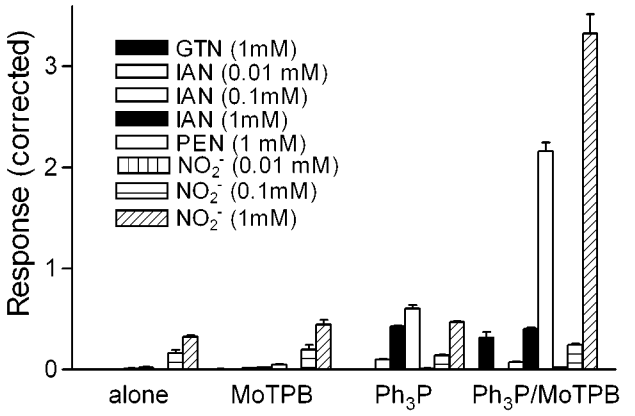


Figure 3. Scaled response of chemiluminescence detector to direct injection of headspace from sealed reaction vessels containing MoTPB (1 mM), Ph_3P (20 mM), and nitrate/nitrite in CH_2Cl_2, maintained at 25 °C, after 10 min reaction. Data are means (± s.e.) of triplicate assays. NO is also observed from Ph_3P/MoTPB/NO_3^-. Response (corrected) is standardized potentiometer response, mV.

kinetically competent intermediates in reduction of nitrates to NO. The requirement for Mo catalysis of the second step in NO release from nitrates is questionable, since nitrites as reaction intermediates would be at lower concentrations at which background reduction to NO is sufficient to account for NO release from GTN (Figure 3).

NO_2^- was quantified as a product of the reduction of GTN by MoTPB/Ph_3P and therefore must be present in the reaction medium and serve as a potential source of NO. However, there is also strong evidence that organic nitrite is an intermediate in nitrate reduction by MoTPB/Ph_3P. Phosphine oxidation was followed by ^{31}P NMR for NO_2^- and IAN in the MoTPB/Ph_3P/CH_2Cl_2 system. Reaction of NO_2^- cleanly converted Ph_3P (δ −5 ppm) to Ph_3PO (δ 25 ppm), whereas in the reaction of organic nitrite, a persistent signal is seen corresponding to $Ph_3P^+N{=}PPh_3$ (δ 22 ppm; characterized by synthesis of an authentic sample; ≤ 20% of total

(23) Zavorin, S. I.; Artz, J. D.; Dumitrascu, A.; Nicolescu, A.; Scutaru, D.; Smith, S. V.; Thatcher, G. R. J. *Org. Lett.* **2001**, *3*, 1113.

(24) Reactions of N and S nucleophiles with nitrates at N and O are known: Hayward, L. D.; Purves, C. B. *Can. J. Chem.* **1954**, *32*, 19. Merrow, R. T.; Cristol, S. J..; van Dolah, R. W. *J. Am. Chem. Soc.* **1953**, *75*, 4259. Reference 23. Artz, J. D.; Yang, K.; Lock, J.; Sanchez, C.; Bennett, B. M.; Thatcher, G. R. J. *Chem. Commun.* **1996**, 927.

products). This phosphine nitrosylation product was also seen as a minor product (<5%) in the reduction of GTN, supporting the highly reactive nitroso-GDN as a reaction intermediate.[25]

MoTPB and other Mo-complexes have been shown to accelerate OAT to Ph_3P from sulfoxide and amine/pyridine-*N*-oxide substrates.[20,21] The initial interaction of P nucleophile with $Mo^{VI}O_2$ is with the $\pi^*(MoO_2)$ orbital, proceeding to P−O bond formation and Mo−O bond cleavage to give $Mo^{IV}O$.[22] OAT from S or N to $Mo^{IV}O$ regenerates $Mo^{VI}O_2$, but Mo^{V} species are also formed. Of the reactions postulated above, the only simple OAT is the reaction of a nitrate with $Mo^{IV}O$ generating $Mo^{VI}O_2$ and an organic nitrite (eq 1a). The latter intermediate and the alternative, NO_2^-, are clearly capable of generating a greater flux of NO than is observed from GTN (Figure 3). Potential alternative intermediates and products include NO_3^-, NO_2, N_2O, NO^-, and higher N,O species; further detailed study of the MoTPB/Ph_3P system is in progress.

The pioneering work of Holm, Enemark, Young, and others on molybdoenzyme models has allowed us to develop a functional model of nitrate biotransformation.[20−22] The major conclusion from this work is the unambiguous demonstration that molybdoenzymes have the catalytic apparatus not only for reductive denitration[26] but also for catalysis of mechanism-based biotransformation of nitrates to generate NO. To the best of our knowledge, this is the first simple system in which a significant flux of NO has been observed from reaction of a classical nitrate such as GTN.[9,27] It is also important that reduction of nitrate to NO does not require anaerobic conditions. There are further important corollaries from this work.

First, the comparison between GTN and TGDN shows that nitrates can be engineered to differentially release NO in response to different reaction conditions and catalysts, opening the way to tissue selectivity for nitrates.[23,28]

Second, NO_2^- cannot be discounted as a biological source of NO and as an intermediate in mechanism-based biotransformation. NO_2^- was central to Ignarro's early hypothesis of nitrate biotransformation, and Mark's later modification.[29] However, NO_2^- was abandoned since physiological concentrations can be high, almost to 1 mM, and aqueous NO_2^- solutions cannot reproduce the NO-mimetic effects of GTN.[30] However, the data presented herein show that NO_2^-, when generated in a hydrophobic environment (modeled by CH_2Cl_2), will break down to NO and that this reaction is accessible to catalysis. This concept can be simply illustrated. In a 1:1 biphasic system of CH_2Cl_2 (MoTPB (1 mM); Ph_3P (20 mM))/aqueous phosphate buffer (pH 7.4, 100 mM), injection of NO_2^- (1 mM) into the aqueous layer generates negligible NO, as determined by headspace chemiluminescence measurement. However, injection of NO_2^- into the organic layer generates $1.4 \pm 0.19\ \mu$M NO after 10 min and 210 ± 40 nM NO in the absence of MoTPB/Ph_3P. NO_2^- is a viable biological source of NO, and further, a $2e^-$ reduction of GTN to generate NO_2^- in a hydrophobic environment and/or proximal to a reducing cofactor clearly represents a potential mechanism-based biotransformation pathway that should be re-examined.

The solubility limits of the MoTPB/Ph_3P system require use of organic solvents that reasonably mimic hydrophobic biological environments, especially relevant for the lipophilic nitrates. To extend their scope toward NO release in biological systems, molybdoenzyme models for use in aqueous solution are being developed.

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(25) Buckell, F.; Hartry, J. D.; Rajalingam, U.; Bennett, B. M.; Whitney, R. A.; Thatcher, G. R. J. *J. Chem. Soc., Perkin Trans. 2* **1994**, 401.

(26) DMSO reductase from *E. coli*, itself, catalyzes the reductive denitration of GTN. Chichirau, A.; Thatcher, G. R. J. Unpublished results.

(27) Artz, J. D.; Thatcher, G. R. J. *Chem. Res. Toxicol.* **1998**, *11*, 1393.

(28) Yang, K.; Artz, J. D.; Lock, J.; Sanchez, C.; Bennett, B. M.; Fraser, A. B.; Thatcher, G. R. J. *J. Chem. Soc., Perkin Trans. 1* **1996**, 1073.

(29) Ignarro, L. J.; Lippton, H.; Edwards, J. C.; Baricos, W. H.; Hyman, A. L.; Kadowitz, P. J.; Gruetter, C. A. *J. Pharmacol. Exp. Ther.* **1981**, *218*, 739. Marks, G. S. *Can. J. Physiol. Pharmacol*. **1987**, *65*, 1111.

(30) Kowaluk, E. A.; Chung, S. J.; Fung, H. L. *Drug Metab. Dispos.* **1993**, *21*, 967. Romanin, C.; Kukovetz, W. R. *J. Mol. Cell. Cardiol.* **1988**, *20*, 389.