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Myoglobin as an Efficient Electrocatalyst for Nitromethane Reduction

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Xenobiotic metabolizing heme enzymes are thought to take a crucial part in the activation of a variety of carcinogens, including nitro compounds, through catalytic electron-transfer reactions, especially under anaerobic conditions. Myoglobin (Mb), as a model heme enzyme, is found to act as an efficient electrocatalyst for the reduction of nitromethane in thin surfactant films on pyrolytic graphite electrodes. The electrocatalytic process is characterized by cyclic voltammetry. The Mb–Fe^{II}–nitrosomethane complex, a possible intermediate in the catalysis, is characterized spectroscopically in the surfactant film on indium tin oxide electrodes. Bulk electrolysis indicates the formation of mainly methylhydroxylamine as an end aqueous product. A rationale for the catalysis invokes the highly reduced Fe^{I} state of myoglobin in surfactant film; the latter engages in efficient inner-sphere electron transfers to the nitro compound coupled to proton transfers.

Introduction

Organic compounds containing nitro groups are widely used substances, with a variety of applications. They are used, for instance, in industry as synthetic intermediates to manufacture dyes as well as some primary chemicals for rubber. This class of compounds is also used as special solvents, in fuel additives, and even as explosives; two famous examples are trinitrotoluene (TNT) and nitroglycerine.

Due to their widespread use, mentioned above, organic nitro compounds end up in our environment through a number of entry points including emissions, wastes, and spills. These compounds also find their way to the human body through direct and indirect exposure, causing a variety of health problems. For all of these reasons, organic nitro compounds are considered a threat to health and the environment, and thus appear on most priority pollutant lists.[1]

Some studies have shown that nitromethane (CH_3NO_2) and some secondary nitroalkanes, such as 2-nitropropane, are carcinogenic after chronic inhalation.[2] Other studies have demonstrated that the reduction of the nitro group of aromatic nitro compounds leads to reactive intermediate species, which interact with DNA, causing irreversible chemical damage to the double helix.[3]

The chemical interaction of aliphatic nitro compounds with heme proteins, including myoglobin and hemoglobin, was thoroughly addressed by D. Mansuy et al.[4] The Mansuy group has shown that various Fe^{III} heme proteins form stable nitrosoalkane complexes after reacting with aliphatic nitroalkane compounds under reducing conditions (Scheme 1).[4] The same complexes can be obtained by the reaction with the corresponding alkylhydroxylamines under aerobic conditions.[5] This type of chemical interaction seems to be quite general and was found to proceed even with some P450-type heme proteins, including nitric oxide synthase (NOS).[6]

Xenobiotic-metabolizing P450 enzymes are thought to take a crucial part in the activation of organic nitro compounds,[7] probably through catalytic electron-transfer reactions in the absence of dioxygen. To gain more insight on the potential

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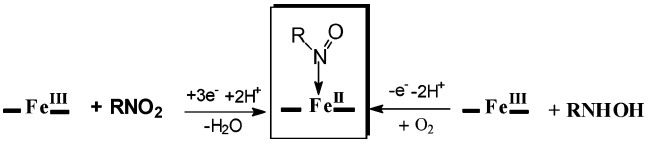
Scheme 1. Heme−Fe^{II}−Nitrosoalkane Complex Formation from Alkylhydroxylamine under Aerobic Conditions and from the Corresponding Nitroalkane under Anaerobic Reducing Conditions



interaction of nitro organic substances with the redox activity of heme enzymes, we investigated the effect of nitromethane as a model compound on the redox behavior of myoglobin as a heme protein.

The rate of heterogeneous electron transfer to myoglobin and a host of other metalloproteins is greatly enhanced in thin films of surfactants, such as the cationic didodecyldimethylammonium bromide (ddab). The procedure is known as the Rusling methodology[8] and allows one to investigate the redox activity of metalloproteins and other nonconventional biological species by direct electrochemistry, i.e., without the use of mediators. Myoglobin (Mb) in ddab films undergoes fast and reversible direct electron exchange with underlying pyrolytic graphite (PG) electrodes.[8,9] The Mb/ddab system was shown to catalyze the electrochemical reduction of many compounds, including ethylene bromide and trichloroacetic acid.[10] The Mb/ddab films were also used to study biomimetic denitrification reactions since they were found to efficiently catalyze the reduction of nitrite (NO_2^-),[11a] nitric oxide (NO),[11b] and nitrous oxide (N_2O).[11c] Herein, we use the surfactant methodology to investigate the interaction of nitromethane and redox heme proteins as modeled by myoglobin.

Experimental Section

Chemicals. Horse heart myoglobin (MW = 17 566) from Sigma was dissolved in acetate buffer, pH 5.5, and passed through Amicon filters (30 000 MW cutoff) to remove large aggregates, and the concentration was then raised to the desired value using a 3000 MW cutoff Amicon membrane. The Mb concentration was estimated by absorption spectroscopy. ddab and nitromethane were purchased from Across Organics. *N*-Methylhydroxylamine was purchased from ICN Biomedicals Inc. Deionized water was obtained on a Barnstead water purification system (specific resistance > 18.2 MΩ cm). All other chemicals were reagent grade and were used as received.

Preparation of Mb/ddab Film. Prior to coating, basal-plane PG electrodes were polished consecutively on 400 grit carbimet disks (Buehler), then on Buehler microcloth, using 0.3 μm alumina. Electrodes were then ultrasonicated in pure water for 30 s, rinsed, and dried in air. The solution of ddab (10 mM) was prepared by dissolving ddab powder in water and sonicating for about 2 h, or until the mixture turned clear. This solution was sonicated again for 5 min right before coating. The Mb/ddab film was prepared by casting 10 μL of 10 mM ddab solution and 10 μL of 0.4 mM Mb on the surface of PG electrode. The modified electrodes were kept in a tightly closed tube, allowing slow water evaporation. The film was then dried in air overnight.

Apparatus and Procedures. All electrochemical experiments were performed in a three-electrode cell on a BAS 100B electrochemical workstation. The Ag/AgCl system was used as the reference electrode on a sidearm connected to the main cell via a luggin. All potentials reported here are versus this reference. A platinum wire was used as the auxiliary, the Mb/ddab-modified PG being the working electrode. PG (A. Ceramics) was cut into cylinders along the basal plane orientation and sealed in a glass tube with epoxy (Huntington Labs). Contact with copper wires was secured with silver epoxy (Huntington Labs). Indium tin oxide (ITO) plates were purchased from Quantum Coating Inc. (Mont Laurel, NJ). Prior to modification with Mb/ddab (vide infra), ITO plates were thoroughly rinsed with deionized water, acetone, and methanol, then ultrasonicated for ca. 5 min in water, rinsed, and finally dried under a N_2 stream. All experiments were performed at room temperature in 100 mM pH 5.5 acetate buffer, containing 100 mM sodium bromide, unless otherwise indicated. The buffer was purged with purified nitrogen for at least 20 min prior to the experiments to remove dioxygen. A nitrogen blanket was then kept over the solution throughout the experiments.

UV−vis absorption spectra were recorded on an Agilent 8453 spectrophotometer. Similar to PG electrode modification, films on transparent conductingITO plates were prepared by depositing a 1:1 (v/v) mixture of 0.4 mM Mb and 10 mM ddab (ITO). The films were kept in a covered Petri dish and then allowed to dry in air before use.

Bulk electrolysis was conducted in 100 mM ammonium acetate pH 5.5 buffer. Mass spectrometry characterization of the compounds was carried out on a Micromass triple quadrupole mass spectrometer. The mass spectrometer was operated in the positive electrospray ionization mode (ESI+). Aliquots from the bulk electrolysis solution were taken at different times during electrolysis, mixed with acetonitrile, 50% (v/v), and then introduced by sample infusion. 2-Nitroethanol was used as an internal standard. Data analysis was performed using Micromass MassLynx v 3.3.

Results

Mb/ddab/PG System. Figure 1a illustrates a typical cyclic voltammetry response of Mb/ddab films on basal PG electrodes in pH 5.5 buffer. Two pairs of well-defined reversible couples are obtained; the first, R1/O1, assigned to the Fe^{III}/Fe^{II}, appears at −0.14 V vs Ag/AgCl, and the second, R2/O2, commonly assigned to the formal Fe^{II}/Fe^{I} couple,[8,11] is shown at −1.03 V vs Ag/AgCl. As expected, no voltammetric peak is observed for the ddab-only modified PG electrodes in the same voltage window (Figure 1b).

Response in the Presence of Nitromethane. When nitromethane **(**CH_3NO_2) is added to the electrochemical cell, a new peak, R3, with significantly higher current appears at about −1.0 V vs Ag/AgCl (Figure 1c). This increase in current is accompanied by disappearance of the Mb−Fe^{II}/Fe^{I} peak and the loss of its reoxidation return. A control experiment on ddab/PG in the absence of Mb shows that CH_3NO_2 does give a reduction wave, but with lower current and at more negative potentials (ca. −1.2 V) (Figure 1d).

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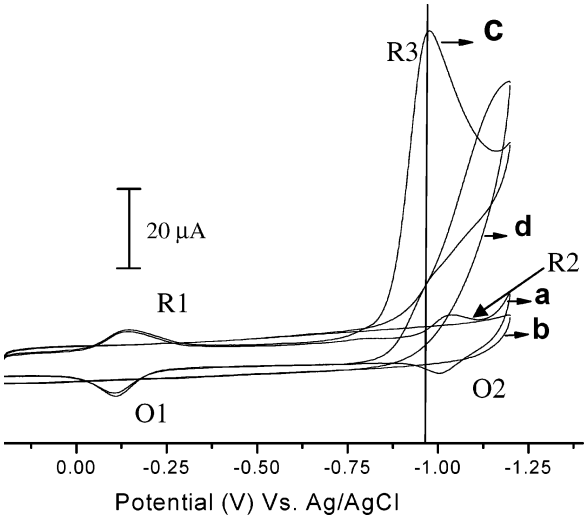

Figure 1. Cyclic voltammograms at 0.15 V/s in pH 5.5 acetate buffer solution containing 0.1 mM NaBr for (a) Mb/ddab/PG film, (b) ddab/PG film, (c) Mb/ddab/PG film with 0.4 mM nitromethane, and (d) ddab/PG film with 0.4 mM nitromethane.

The current of the new wave, R3, increased with increasing concentration of nitromethane, as clearly shown in Figure 2a. The contribution of direct reduction of nitromethane is expected to be very small under catalytic conditions.[12] The ratio of the peak current at R3 in the presence of nitromethane, I_c, and that of the first reduction peak at R1, I_d, was monitored as a function of CH_3NO_2 concentration. Figure 2b shows that I_c/I_d increases with increasing nitromethane concentration. The inset in Figure 2b shows a typical double-reciprocal analysis graph, yielding an estimated Michaelis constant, K_m, of about 1.4 mM.

We also examined the effect of the scan rate on the response of Mb/ddab in the presence of nitromethane. As a result, we found that the current ratio, I_c/I_d, decreases as the scan rate increases (Figure 3).

The effect of nitromethane was investigated at different pHs. Figure 4a shows cyclic voltammetry measurements carried out as a function of pH. The current and peak potential of the wave R3 were found to depend strongly on the pH. In fact, the I_c/I_d current ratio decreases significantly as the pH is increased in the $5.5 < pH < 10$ range, as shown in Figure 4b.

Voltammetry in the Presence of *N*-Methylhydroxylamine, CH_3NHOH. Stable heme−Fe^{II}−nitrosoalkanes (Scheme 1) are known to form when alkylhydroxyamines are reacted with Fe^{III} heme proteins.[4,5] As methylhydroxylamine is one possible product or intermediate in the reduction of nitromethane, we examined the cyclic voltammetry response of Mb/ddab/PG in the presence of methylhydroxylamine. The response of the electrode in the voltage window

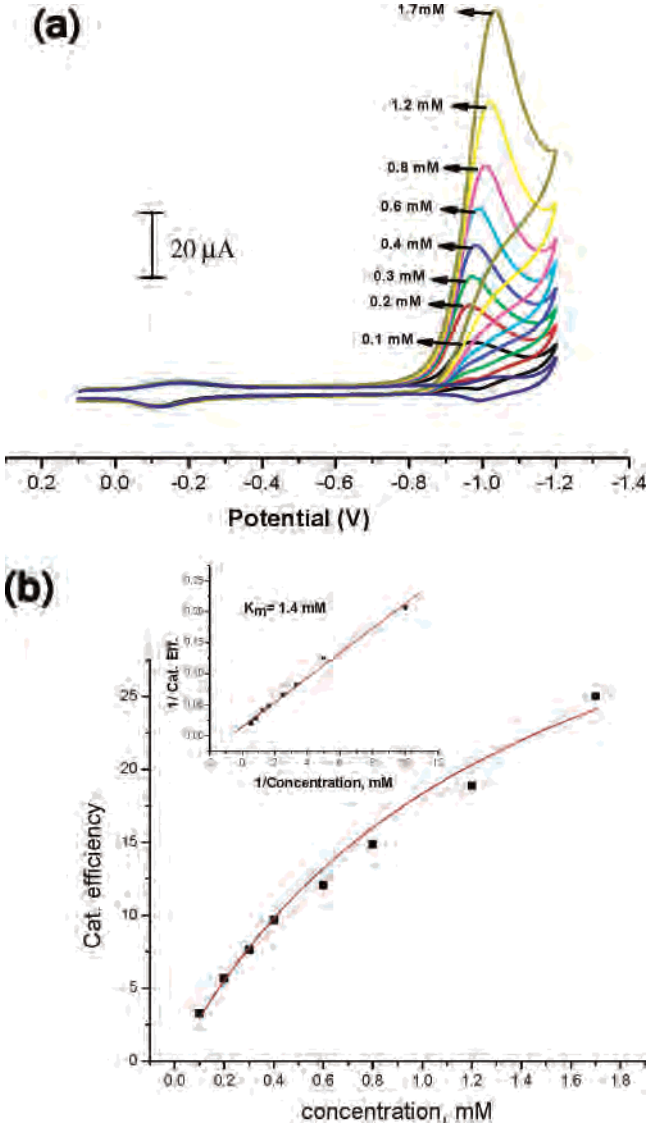

Figure 2. (a) Voltammograms of Mb/ddab in pH 5.5 for different nitromethane concentrations from 0.1 to 1.7 mM; the scan rate is 0.15 V/s. (b) Catalytic efficiency, expressed as I_c/I_d (see text), as a function of nitromethane concentration at 0.15 V/s; the data are fit to a Michaelis−Menten-type equation with $K_m = 1.4$ mM.

corresponding to the first Mb couple R1/O1 is essentially the same before and after addition of methylhydroxylamine.[13a] Also, an electrode modified with a preformed sample of Mb−Fe^{II}−nitrosomethane,[13b] the product of reaction of methylhydroxylamine and metmyoglobin (i.e. Fe^{III}−Mb) under aerobic conditions, gives a reversible couple identical (same formal potential) to that observed when CH_3NHOH is added, i.e., essentially Mb−Fe^{III}/Fe^{II} redox couple.[13c] At more negative potentials, however, one observes a drastic change of the Mb/ddab/PG in the presence of CH_3NHOH, as illustrated by the cyclic voltammogram shown in Figure

(12) Any contribution of the direct reduction current of nitromethane in ddab is expected to be very small. Subtraction of voltammograms in ddab/PG at different concentrations of nitromethane from catalytic voltammograms recorded on Mb/ddab/PG would actually overestimate the direct contribution. In fact, under catalytic conditions, as described, the catalytic consumption of nitromethane is kinetically faster than direct reduction and is such that the substrate concentration is virtually zero at any moment at the electrode surface in the presence of myoglobin loaded in the film.

(13) (a) A voltammogram demonstrating this fact is given in Supporting Information. (b) The complex was formed by reaction of myoglobin with nitromethane in the presence of excess dithionite and was purified on size-exclusion column. The Mb−nitrosomethane was found to be very stable, which allowed us to modify PG electrodes as described in ref 11b. The stability of the preformed complex in the film was checked by UV−vis spectroscopy. (c) Also see Supporting Information for experimental evidence.

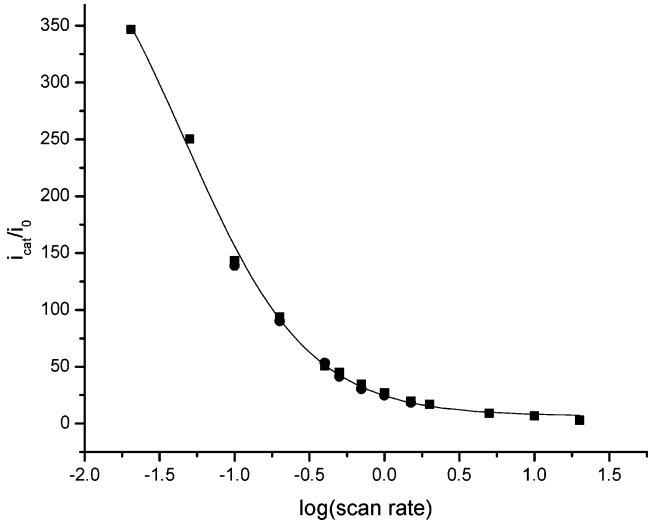

Figure 3. Influence of scan rate on catalytic efficiency, I_c/I_d (see text), for a Mb/ddab film in pH 5.5 buffer containing 0.4 mM nitromethane. Note that catalytic efficiencies on the same modified electrode can be faithfully reproduced from separate experiments (● and ■ in this case).

5a. A new reduction wave, R4, is observed at a potential more negative (i.e. −1.39 V) than that of the wave, R3, obtained in the presence of nitromethane. The same wave, R4, is observed in the presence of CH_3NO_2 if the voltage is scanned negative to R3 (Figure 5b).

UV−Vis Spectroscopy. As seen with numerous of Fe^{III} heme proteins,[5,14] Mb−Fe^{III} (Soret at 409 nm, pH 5.5) reacts with methylhydroxylamine in aerobic solution to give a stable Mb−Fe^{II}−nitrosomethane complex (Soret at 424 nm, pH 5.5). We examined if the same reaction occurs in the Mb/ddab films on ITO slides immersed in pH 5.5 acetate buffer. Addition of 2 mM methylhydroxylamine in the solution causes a gradual absorbance increase at 424 nm concomitant with an absorbance decrease at 409 nm, giving an isosbestic point at about 416 nm (Figure 6). The multiple-trace spectrum clearly shows that myoglobin in ddab films reacts with methylhydroxylamine to give a species with Soret absorbance identical to that of the Mb−Fe^{II}−nitrosomethane complex characterized in solution. Similar experiments with nitromethane in the presence of dithionite also show the formation of the 424 nm-absorbing species. On the other hand, in controls without reductant, the Mb Soret in ddab films does not change in the presence of nitromethane.

Discussion

Rusling's surfactant-film methodology provides a valuable approach to study the direct electrochemical behavior of metalloproteins, including heme proteins, such as myoglobin.[8,10,15] The voltammetric response of Mb/ddab in pH 5.5 acetate buffer is dramatically changed in the presence of nitromethane. While no change is seen at the level of the Fe^{III}/Fe^{II} couple, a new wave, R3, appears at a potential close to the formal Fe^{II}/Fe^{I} redox couple. R3 has relatively large

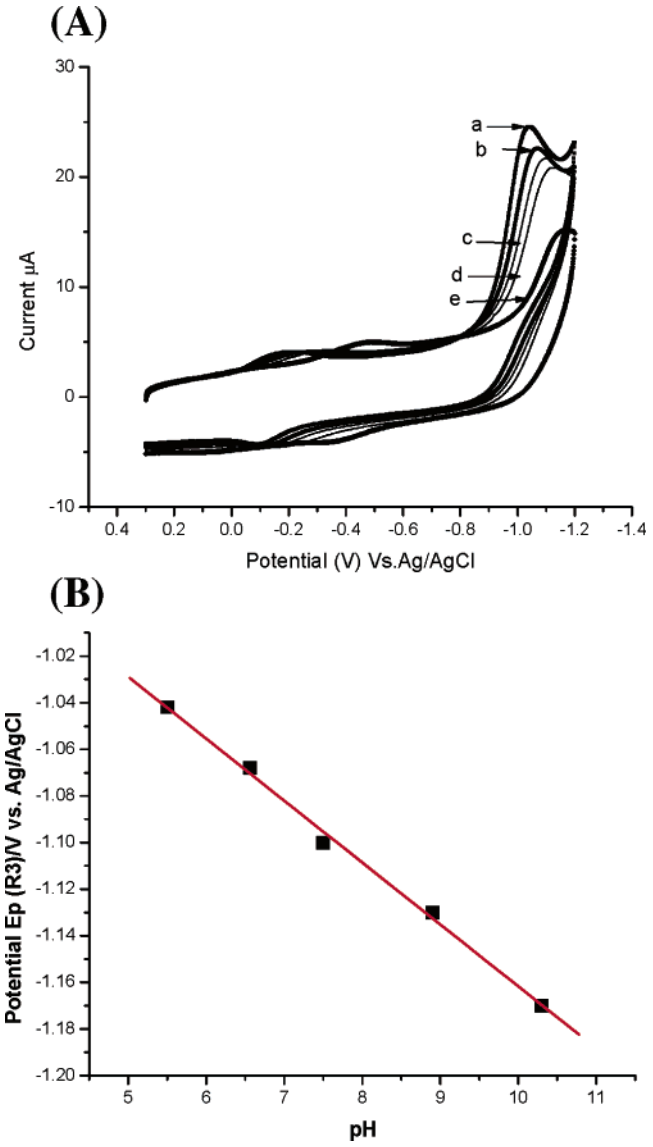

Figure 4. (A) Catalytic reduction of 0.2 mM nitromethane by Mb/ddab at (a) pH 5.5, (b) 6.56, (c) 7.5, (d) 8.9, and (e) 10.3. (B) Peak potential of R3 as a function of pH; scan rate = 0.15 V/s.

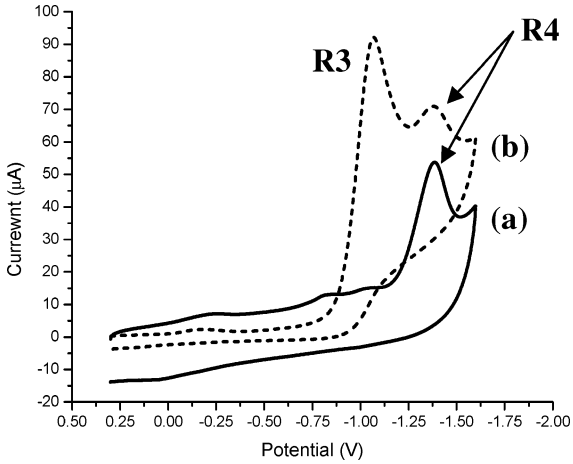

Figure 5. (a) Cyclic voltammogram of Mb/ddab/PG at 0.15 V/s in the presence of 2 mM methylhydroxylamine in pH 5.5 acetate buffer (—). (b) Cyclic voltammogram of Mb/ddab/PG in the presence of 2 mM nitromethane (- - -).

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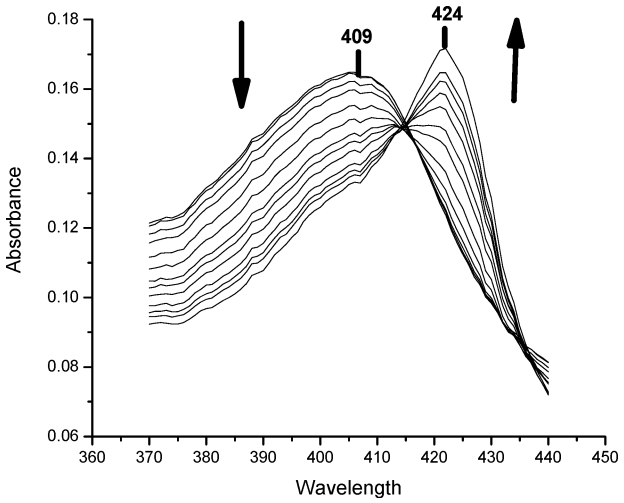
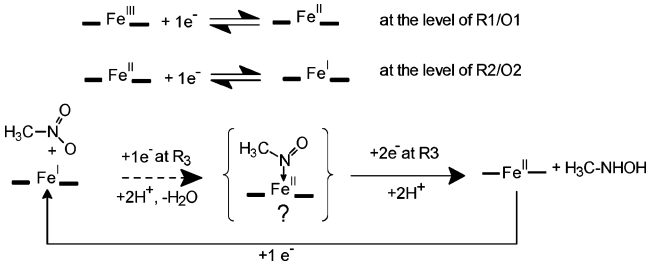


Figure 6. Evolution of the absorption spectrum of Mb/ddab on ITO after addition of 2 mM methylhydroxylamine in pH 5.5 acetate buffer. Spectra were recorded every 2 min.

current and causes a loss of reversibility of the Fe^{II}/Fe^{I} couple (Figure 1c). These changes are not observed in the absence of Mb. This behavior is consistent with Mb-mediated electrocatalytic processes at the formal potential of the Fe^{II}/Fe^{I} redox couple.[8,11,16] The peak shape of the catalytic wave, R3, is a result of substrate depletion in the film during turnover and subsequent replenishment by diffusion from the bulk. This is inevitable in the absence of forced mass transport, for instance, using a rotating disk electrode setup. However, this is not possible due to the limited mechanical stability of ddab films under forced convection. The catalytic efficiency of the observed process is expressed as the ratio, I_c/I_d, i.e., the catalytic current at R3 normalized to the reduction current of R1/O1.[11c,16] Consistent with an electrocatalytic process triggered within the cyclic voltammetry time scale, the ratio I_c/I_d decreases as the scan rate increases (Figure 3). It is worth mentioning that although the efficiency decreases at shorter times (i.e., higher scan rates), the reversibility of the Fe^{II}/Fe^{I} couple is not regained, even for a scan rate as high as 20 V/s.

That the peak potential of the first Mb wave, R1, is not affected by addition of nitromethane indicates that the latter does not bind to the Fe^{II} form of Mb. The electrocatalysis observed involves, therefore, the formal Fe^{I} state. The onset of the elecrocatalytic reduction wave, R3, and its peak potential are clearly positive compared to the Fe^{II}/Fe^{I} couple, indicating a significant interaction (i.e., chemical catalysis)[16,17] of nitromethane with the Mb−Fe^{I} catalyst followed by a rapid and irreversible reaction regenerating the catalyst at the formal potential of the second wave. The proposed inner-sphere interaction between the Fe heme and nitromethane requires that the size of the substrate allows access to myogobin's active site. In this regard, a large body of published work shows that, among nitroalkanes, nitromethane is easily accommodated by the distal site of myoglobin, as well as a host of other heme proteins.[4−6,14] In

Scheme 2. Catalytic Scheme for the Electroreduction of Nitromethane Mediated by Mb/ddab System[a]



[a] The question mark is to emphasize that this scheme only *assumes* the intermediacy of the nitrsoalkane complex. Its reduction wave is not detected yet.

agreement with the published work, evidence from UV−vis spectroscopy experiments in the current work clearly shows the formation of the well-known nitrosomethane−myoglobin complex from nitromethane under reducing conditions (see Results section).

To gain more insight on this catalytic process we carried out bulk electrolysis experiments on large PG plates modified by Mb/ddab. Mass spectrometry analysis of aqueous products indicates the formation of mainly methylhydroxylamine.[18] Together these results suggest that Mb/ddab acts as an efficient and selective catalyst, reducing nitromethane to methylhydroxylamine at the formal potential of the Mb−Fe^{II}/Fe^{I} couple. The electrochemically driven catalytic process is summarized in Scheme 2.

The process of reduction of nitromethane to methylhydroxylamine requires four electrons and four protons.[19] As implied in Scheme 2, protons play a critical role in driving elemental steps of the overall catalysis. This is consistent with the experimental facts observed when the catalytic current at R3 is monitored as a function of pH. Indeed, the catalytic efficiency drops significantly upon increasing the pH from 5.5 to 10.3 (see Supporting Information, S3). The peak potential of R3 also shifts negatively as the pH increases, as expected for proton-dependent processes. Figure 4b shows a gradient close to ca. −30 mV/pH, which indicates that the elementary steps determining the turnover include two electron-transfer steps coupled to one proton transfer. On the other hand, the proposed nitrosomethane intermediate in Scheme 2 is obtained via a formally $2e^-/2H^+$ process. The experimentally observed gradient of ca. −30 mV/pH may suggest that only one proton-transfer step may be turnover-determining.

The catalytic current increases as a function of the substrate concentration, as evidenced by data in Figure 2a; however, at concentrations higher than ca. 0.75 mM, the beginning of saturation kinetics is observed (Figure 2a). In this context, double-reciprocal analysis (Lineweaver−Burk plot) of the data yields a Michaelis−Menten constant, K_M, of 1.4 mM (inset in Figure 2b). The peak potential of the catalytic wave, R3, shifts negatively with nitromethane concentration; a

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gradient of −82 mV/decade is observed (Supporting Information, S2). Since our data also show that the Mb−Fe^{II} state does not bind nitromethane, the rationale of the negative shift of R3 with concentration in terms of a potential mechanism is not obvious at this point.

The nitrosoalkane ligands, R−N═O, are isoelectronic with dioxygen, O_2, but the heme−Fe^{II}−nitrosoalkanes complexes are more stable than the heme−Fe^{II}−O_2 complexes. They are known to form by reaction of Fe^{III}−heme and nitroalkanes in the presence of chemical reductants (e.g. dithionite).[4,5] As assumed in our working Scheme 2, the Mb−Fe^{II}−nitrosomethane complex is most likely an intermediate in our electrochemically driven reduction of nitromethane, mediated by Mb. Therefore, we carried out a spectroscopic characterization of this complex in ddab films and, at the same time, attempted to examine its electrochemistry. To this end, films of Mb/ddab were prepared on transparent ITO electrodes to allow both electrochemical and spectroscopic characterizations. Mb/ddab/ITO was first electrochemically tested in the range of the first couple R1/O1 (Figure 7a). UV−vis spectroscopy was then taken before and after the reaction with methylhydroxylamine under aerobic conditions. Difference spectra clearly show the loss of the Soret band of Mb−Fe^{III} and formation of the Mb−Fe^{II}−nitrosomethane species with Soret at ca. 424 nm (Figure 7b). Transfer of the Mb−Fe^{II}−nitrosomethane/ddab/ITO electrode back to the electrochemical cell gave essentially the same redox couple characterizing the Fe^{III}/Fe^{II} of Mb (Figure 7c), identical to the redox couple obtained on PG electrodes.[13c] This is most likely due to an electrochemically driven oxidation of the Mb−nitrosomethane complex prior to potential scanning. In fact, it has been reported that the nitrosoalkane−Fe^{III}(Heme) bond is very weak[12] and is expected to break when the nitroso−Fe^{II} form is oxidized. Along the same lines, heme−nitrosoalkanes are known to decompose in the presence of chemical oxidants, releasing the nitrosoalkanes, R−N═O, and the heme−Fe^{III}.[14] Attempts to electrochemically characterize the Mb−nitroso complex (single turnover experiments) at more negative potentials to prevent oxidation were so far unsuccessful; however, more efforts in this direction are still underway.

Since bulk electrolysis experiments showed the formation of methylhydroxylamine, we examined the response of Mb/ddab in the presence of methylhydroxylamine (CH_3NHOH). While the Fe^{III}/Fe^{II} redox couple of Mb remains unaffected upon addition of CH_3NHOH,[13a] a dramatic change occurs at more negative potentials (Figure 5a). A reduction wave, R4, is observed, with a peak potential more negative than that of the catalytic reduction of nitromethane R3. One obtains the same electrochemical wave in situ if the voltage window is extended to more negative potentials during the electrocatalytic reduction of nitromethane by Mb/ddab/PG (Figure 5a and b). The R4 wave is electrocatalytic (with higher current) and does not correspond to the direct reduction of CH_3NHOH in ddab films, as confirmed by controls; in fact, in similar conditions (but in the absence of Mb), the direct reduction occurs but is very sluggish, with much lower currents, and, most importantly, occurs at more negative potentials (ca. −1.5 V, just before the discharge wall in our conditions). Whether this second Mb-mediated electrocatalytic reduction corresponds to reductive catalysis of CH_3NHOH to yield the ultimate reduced product, methylamine, remains to be addressed. At this point, however, we did notice that bulk electrolyses at slightly more negative potentials than R3 give variable amounts of the reduction product, methylamine, as detected by mass spectrometry.

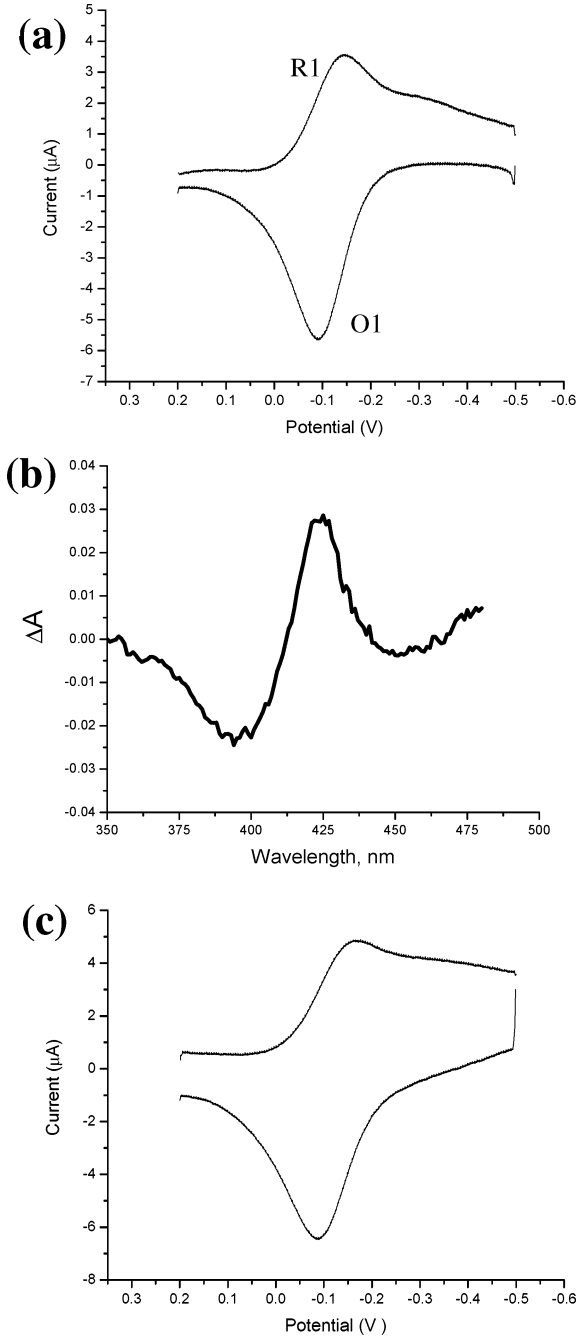

Figure 7. (a) Cyclic voltammogram at 0.15 V/s of the Mb/ddab film in acetate buffer, pH 5.5; the ITO background current was subtracted. (b) Difference spectrum of the Mb/ddab/ITO film before and after reaction with 2 mM methylhydroxylamine in aerobic pH 5.5 acetate buffer, showing formation of the Mb−nitroso complex. (c) Cyclic voltammogram at 0.15 V/s of the Mb/ddab/ITO film in acetate buffer, pH 5.5, after in situ formation of the Mb−nitroso complex, as shown by the difference spectrum (b) above; the ITO background was subtracted.

Most importantly, however, the second wave, R4, observed in situ during the catalytic reduction of nitromethane on Mb/ddab/PG, indirectly proves methylhydroxylamine as the product of the catalysis at the level of R3. Closer investigations of product distribution and faradaic yields of bulk electrolyses at different potentials are now underway.

Conclusion

In conclusion, Mb/ddab/PG acts as an efficient catalyst, mediating the selective reduction of nitromethane, as a model organic nitro compound, to the corresponding methylhydroxylamine. To some degree, the system can be seen as a mimic of enzymatic nitro compound reductases. Xenobiotic metabolizing P450s are also known to act as reductases for these compounds under anaerobic conditions,[20] leading to reactive and possibly carcinogenic species. We are now conducting comparative investigations using a number of other nitro compounds with the Mb/ddab system, as well as with other heme proteins including P450s-type proteins.

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Supporting Information Available: Voltammograms of Mb/ddab and PG electrode, peak potential of catalytic wave R3, and catalytic efficiency measured on R3 as a function of pH. This material is available free of charge via the Internet at http://pubs.acs.org.

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