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Direct Electron Transfer to the Oxygenase Domain of Neuronal Nitric Oxide Synthase (NOS): Exploring Unique Redox Properties of NOS Enzymes

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Nitric oxide (NO) is a surprisingly important molecule if one considers its small size, simple structure, and the attention it was given in a number of fields, including physiology and medicine, environmental chemistry, and fundamental catalysis.[1,2] The molecule was mostly known as an insidious poison gas; hence, it was considered far from having any role in living systems. Ironically, however, evidence accumulated over the years pointing to important roles of NO in a myriad of physiologic processes, including regulatory cardiovascular mechanisms, neurotransmission, and immune host-defense.[3] NO is produced in vivo by nitric oxide synthases (NOSs). The reaction takes place on an Fe-heme active center; it is now well established that it proceeds in two steps: the first is converting L-arginine to N^{ω}-hydroxy-L-arginine, which, in the second step, is further oxidized to L-citrulline, to liberate one equivalent of NO.[4] Oxygen is a cosubstrate in the reaction.

The family of NOS enzymes currently contains three isoforms: neuronal NOS (nNOS), inducible NOS, (iNOS), and endothelial NOS (eNOS).[5] The general NOS structure[5,6] contains a reductase domain with binding sites for NADPH (electron source), as well as flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), and an oxygenase domain containing a *P450-type* (i.e. Fe-thiolate) heme. The substrate, L-arginine, and a cofactor, (6R)-5,6,7,8 tetrahydrobiopterin (H_4B), both bind near the heme center in the oxygenase domain.[7−9]

A number of issues still remain unclear regarding the mechanism of NO synthesis by NOSs.[5] The postulated mechanism has some general similarities as well as striking differences with P450 monooxygenases.[5,6] Central to this unique NOS catalysis is the puzzling fact that the NOS heme supports distinct reactivity features from the first to the second step of NO biosynthesis.[10,11] The profound reasons for these differences in the two steps of the cycle are still unclear; however, they are suspected to be related to modulated redox properties of the heme active site.[10,11] Although the iron coordination in the NOS active site is similar to that in other cytochromes P450, the heme environment is structurally distinct, and it is speculated that the structure is uniquely tuned to support the NOS function. Careful investigation of redox properties of Fe-heme in NOS is therefore fundamentally important and would contribute to our understanding of the NOS catalysis.

Voltammetric methods based on direct electron transfer to metalloproteins are gaining extensive ground in investigating enzyme structure/function. Electron-transfer reactions are very often driving forces for coupled chemical reactions making up complex functions in biological processes. The traditional equilibrium methods such as potentiometric titrations are not suited to study these dynamic systems, which often involve transient intermediates. Voltammetric methods based on direct electrochemistry (no mediators) are better suited not only to resolve kinetics but also to determine the thermodynamics of elementary steps in a complex enzymatic function.[12,13]

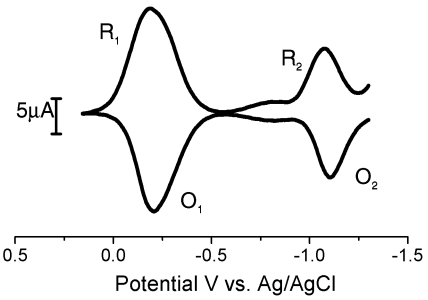

Figure 1. SW voltammetry of nNOSoxy (forward and reverse current contributions to the net SW voltammogram (not shown); scan performed from +0.2V toward negative potentials); background subtracted, $f = 100$ Hz, step: 4 mV, amplitude: 25 mV) in ddab film, pH 7 phosphate buffer.

Direct electron exchange between metalloproteins and electrodes depends on a number of factors, including the purity of sample, favorable interaction between the protein and the electrode surface, and the *right* orientation of the protein's redox active site versus the electrode at the time of an electron-transfer event. Immobilization of proteins on electrodes often enhances rates of heterogeneous electron transfer and allows fast electrochemical measurements. We use here the Rusling's surfactant methodology[14] as applied to purified heme oxygenase domain (MW = ∼80 kD) of neuronal NOS (nNOSoxy),[15] to study redox properties of the heme active site of this important enzyme. The surfactant procedure, which consists of embedding the redox-active species in bilayered film of a surfactant such as didodecyldimethylammonium bromide (ddab), is versatile and has been applied to a number of metalloproteins.[16−18] Figure 1 shows typical forward and reverse current contributions to the square-wave voltammogram (SW)[19] of nNOSoxy/ddab on a pyrolytic graphite (PG) electrode in pH 7 phosphate buffer.[17] Two reversible redox couples are observed; R_1/O_1 at −197 mV and R_2/O_2 at −1092 mV. The first wave R_1/O_1 corresponds to Fe^{III}/Fe^{II} redox couple of nNOSoxy, whereas R_2/O_2 (*vide infra*) represents a second reduction of nNOSoxy in this lipid film environment. UV−vis studies indicate that the native structure of NOSoxy is maintained in the surfactant film.[18] Substrate arginine and cofactor H_4B are known to cause a characteristic low-to-high spin shift of the iron-heme in solution.[6] Similar behavior is observed for nNOSoxy in ddab surfactant film as evidenced by difference spectra in the Soret region.[20a] The shift indicates that substrate and cofactor still recognize their binding sites in immobilized nNOSoxy and demonstrates that analytes in solution can access nNOSoxy proteins in ddab films.

The observed second reduction in ddab films of nNOSoxy with P450-type cysteine-thiolate ligation may reveal unique structural features of the heme in NOSs.[7b,20b]

The effect of binding of cofactor H_4B to nNOSoxy on its redox behavior in ddab films was studied. We found that the Fe^{III}/Fe^{II} heme reduction potential (first wave) moves ca. $\Delta E = 100$ mV

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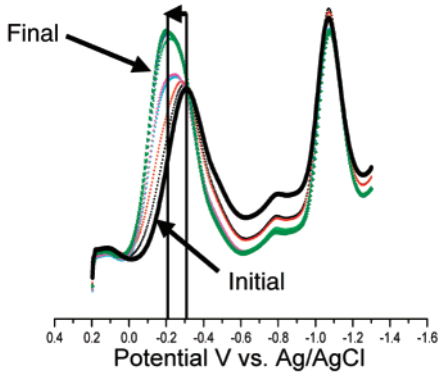

Figure 2. Effect of H_4B (10 μM) on square wave voltammetry of nNOSoxy in pH 7 phosphate buffer; forward scan minus backward scan, with peak giving midpoint potential,[21] over a time course of ca. 10 min after addition of H_4B. Arrows point to initial and final scans.

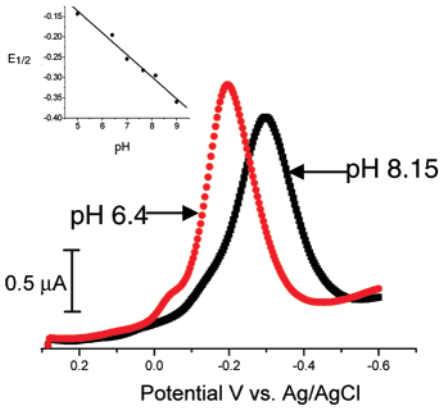

Figure 3. Typical square wave voltammograms at pH 6.4 and 8.15, illustrating the pH effect on the formal potential of the first reduction of nNOSoxy in ddab film. (Inset) pH dependency from pH 5 through 9.

toward positive potentials upon H_4B binding, Figure 2. This indicates that H_4B has a higher affinity for the reduced (Fe^{II}) form of nNOSoxy. Our direct electrochemical findings seem to correlate with the low-to-high spin shift measured by UV−vis for nNOSoxy upon substrate (arginine) and/or cofactor (H_4B) binding. It is often thought that the low-to-high spin shift increases the reduction potential, and thus acts as a gate to the NOS catalytic cycle, by facilitating the first electron transfer.[6]

Electron transfer in biological systems is often accompanied by proton movement. One of the merits of the film methodology is that a prepared electrode can be easily transferred from one medium to another. Therefore, effects of parameters such as pH, which affect biological electron transfer, can be easily investigated. Figure 3 illustrates a preliminary pH-dependence study conducted on the Fe^{III}/Fe^{II} redox couple of nNOSoxy. The inset in this Figure shows a plot of $E_{1/2}$ vs pH. The plot is linear between pH 5 and 9, with a slope of −54 mV/pH unit, which is close to the value known for reduction processes involving one electron and one proton.[22] This behavior is of particular interest in the case of NOS proteins; in fact, the distal pocket of the heme is overall hydrophobic and lacks obvious proton-donating residues[7a] except when the substrate arginine is present. The proton-coupled electron transfer observed in this study may speak to yet another unknown process by which the NOS structure controls electron transfers to the heme active site.

We are using the NOSoxy/ddab system on PG electrodes to study more closely how the redox behavior of NOS heme is modulated by pH as well as by binding of substrate and cofactor. We are also pursuing studies to understand oxygen activation by nNOSoxy/ddab/PG, as it relates to catalytic NO biosynthesis, and how redox properties of the heme in NOSoxy may affect this activation process.

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Supporting Information Available: Difference spectra in the Soret region of NOSoxy in ddab film immersed in pH 7 buffered solution before and after addition of 5 mM Arg. This material is available free of charge via the Internet at http://pubs.acs.org.

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