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Original article

The tetrahydrobiopterin radical interacting with high- and low-spin heme in neuronal nitric oxide synthase – A new indicator of the extent of NOS coupling

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ABSTRACT

Reaction intermediates trapped during the single-turnover reaction of the neuronal ferrous nitric oxide synthase oxygenase domain (Fe(II)nNOS$_{OX}$) show four EPR spectra of free radicals. Fully-coupled nNOS$_{OX}$ with cofactor (tetrahydrobiopterin, BH_4) and substrate (L-arginine) forms the typical BH_4 cation radical with an EPR spectrum ~4.0 mT wide and hyperfine tensors similar to reports for a biopterin cation radical in inducible NOS$_{OX}$ (iNOS$_{OX}$). With excess thiol, nNOSox lacking BH_4 and L-arg is known to produce superoxide. In contrast, we find that nNOS$_{OX}$ with BH_4 but no L-arg forms two radicals with rather different, fast (~250 μs at 5 K) and slower (~500 μs at 20 K), electron spin relaxation rates and a combined ~7.0 mT wide EPR spectrum. Rapid freeze-quench CW- and pulsed-EPR measurements are used to identify these radicals and their origin. These two species are the same radical with identical nuclear hyperfine couplings, but with spin-spin couplings to high-spin (4.0 mT component) or low-spin (7.0 mT component) Fe(III) heme. Uncoupled reactions of nNOS leave the enzyme in states that can be chemically reduced to sustain unregulated production of NO and reactive oxygen species in ischemia-reperfusion injury. The broad EPR signal is a convenient indicator of uncoupled nNOS reactions producing low-spin Fe(III) heme.

1. Introduction

Nitric oxide synthase (NOS) is a homodimeric, heme-containing, mono-oxygenase that produces nitric oxide (NO), a biomolecule important in a number of physiological and pathophysiological processes. The three NOS isoforms in mammals are: constitutive nNOS and eNOS, producing nanomolar amounts of NO for signaling; and inducible iNOS, producing micromolar amounts in the immune response. These isoforms share 50–60% sequence identity and are composed of a heme-containing oxygenase domain (NOS$_{OX}$) and a flavin-containing reductase domain linked by a calmodulin binding site. The three isoforms are expressed in different tissues and are regulated differently [1].

Each NOS isoform converts its L-arginine (L-arg) substrate to NO using a tetrahydrobiopterin cofactor (BH_4, Scheme 1) in a series of tightly-coupled reactions. In several common pathological conditions, the reactions become uncoupled, producing reactive oxygen species (ROS): superoxide and/or hydrogen peroxide in place of NO [2–7]. For example, during ischemia caused by stroke or infarct, tissues become highly reduced because cytochrome *c* oxidase can no longer dispose of electrons to O_2. Consequently, NOS$_{OX}$ may be reduced to its substrate-free Fe(II) heme state; a state rigorously avoided under normal conditions. When reperfusion restores O_2 to the tissue, the incoming O_2 reacts with the reduced state of NOS to produce ROS [8] and may continue to do so as long as the tissue can reduce NOS$_{OX}$. Repeated cycles of unregulated reduction and ROS production could cause major

Abbreviations: arg, arginine; BH_4, tetrahydrobiopterin; CW, continuous wave; ED-EPR, echo-detected EPR; ENDOR, electron nuclear double resonance; EPR, electron paramagnetic resonance, also known as ESR or electron spin resonance; HYSCORE, hyperfine sublevel correlation spectroscopy; mT, milliTesla; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; NOS, nitric oxide synthase; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; nNOS, neuronal nitric oxide synthase; NOS$_{FQ}$, freeze quenched NOS; NOS$_{OX}$, resting oxygenase domain of NOS; RFQ, rapid freeze quench; T, Tesla; T_M, phase memory time or the characteristic time for decay of the two-pulse electron spin echo; T_1, spin-lattice relaxation time

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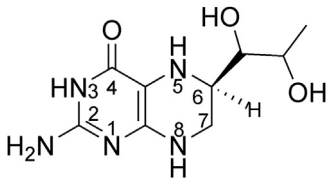
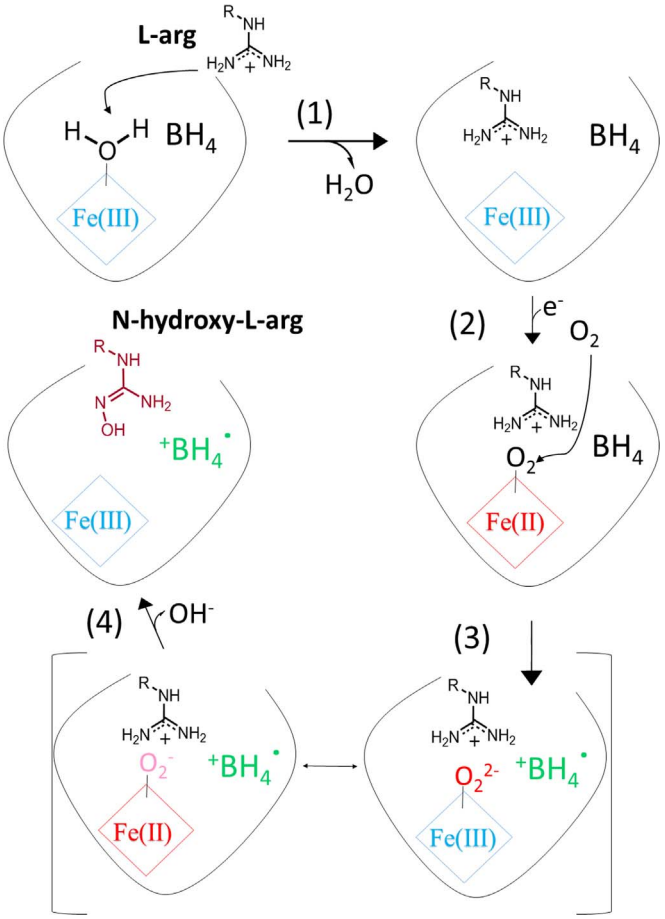


Scheme 1. Structure of BH_4.



Scheme 2. The first mono-oxygenation reaction cycle in NOS_{OX} with key steps numbered. The resting state of the enzyme in the upper, left-hand corner has a hexa-coordinate (Fe(III) heme) with an axial water ligand. Its EPR spectrum has g-factors between ~1.8 and 2.6, typical of a low-spin (Fe(III) heme)-thiolate [9]. After L-arg binding in step 1 and at the end of the first cycle after step 4, there is no axial water ligand and the penta-coordinate (Fe(III) heme)-thiolate has an EPR spectrum with g-factors spanning the 1.8–8.0 range typical of high-spin heme. Other (Fe(III) heme) intermediates in this scheme (and similar states in side reactions) have lifetimes much shorter than the reaction times in the RFQ experiments and are not be present in our samples.

tissue damage, the so-called reperfusion injury.

NOS behaves like a self-sufficient P450-like enzyme. NO is formed in a pair of sequential mono-oxygenase reaction cycles in NOS_{OX}. In the first cycle, Scheme 2, L-arg is hydroxylated to produce N-hydroxy-L-arginine using O_2 and two reducing equivalents. One reducing equivalent comes from NADPH in the reductase domain but the other electron, in contrast to classical P450 enzymes, comes from the BH_4 bound in NOS_{OX}. The second oxygenation cycle uses an additional reducing equivalent and another O_2 to convert N-hydroxy-L-arginine into NO and L-citrulline, and still uses BH_4 for a 2nd reducing equivalent.

The first reaction cycle is well characterized under normal conditions [10–13]. It starts in step (1) when L-arg binds to oxidized NOS_{OX} and displaces the water serving as the sixth, axial ligand of the low-spin, Fe(III) heme, raising the heme redox potential. The heme becomes high-spin and is rapidly reduced in (2) to Fe(II) heme by the reductase domain and immediately binds O_2. The resulting (Fe(II) heme-O_2) is rapidly reduced in (3) by the bound BH_4 cofactor. If reduction by BH_4 is delayed, the (Fe(II) heme-O_2) can decompose to produce superoxide or hydrogen peroxide, due to the very hydrophilic heme distal pocket. Otherwise, the normal reduction step produces an intermediate often described as a hybrid, indicated by square brackets, between (Fe(II) heme-O_2^-) and (Fe(III) heme-O_2^{2-}). This intermediate may react directly with substrate or it may eliminate water to form a ferryl heme (Fe(IV) heme=O) and then transfer its oxygen to the L-arg substrate in (4) [11]. The NOS_{OX} is left containing N-hydroxy-L-arginine and Fe(III) heme, poised to start the second reaction cycle. The BH_4 cofactor is a free radical, but is soon reduced. The strict sequence of these reactions is enforced under normal conditions by several tactics that prevent heme reduction in the absence of substrate. Rapid freeze-quench (RFQ) electron paramagnetic resonance (EPR) spectroscopy in iNOS identified the BH_4 radical through ^{15}N labelling at the N5 position [14]. Subsequent EPR studies found the same BH_4 radical in all isoforms [15–18] as the BH_4 radical cation [15] and we find no new evidence disputing that assignment.

Crystal structures of NOS_{OX} show BH_4 bound near the heme along the homodimer interface [19–21]. The BH_4 is sandwiched between two aromatic amino acids and makes three hydrogen bonds with the heme propionate group (from N3, from N2 and, via a bridging water, from O4 of the BH_4), Fig. 1. The hydrogen bonds provide an electron transfer path from BH_4 to the heme and they stabilize the BH_4 radical cation. However, BH_4 is too far from the (Fe(IV) heme=O) for the activated oxygen to attack and modify it. Yet, BH_4 does form a triad with heme and L-arg in an extended, hydrogen-bonded network that rigidifies the active site scaffolding and helps regulate the axial water ligation controlling the high/low-spin and redox states of the heme. A mutual enhancement of the binding of each element of the triad also guides the relative orientation of O_2 and L-arg which is crucial for ensuring the high fidelity of the monooxygenation reactions.

Reactions of $nNOS_{OX}$ that occur under pathological conditions are much less studied or understood although they can have a major impact on human health, for example, in ischemia-reperfusion injury. We have used single-turnover, RFQ EPR after mixing pre-reduced nNOSox (Fe(II)NOS_{OX}) with O_2 to study radical species during the first cycle of NOS catalysis under pathological conditions [8,22,23]. We found the expected BH_4 radical cation when both BH_4 and L-arg were bound to NOS_{OX} as Fe(II)NOS_{OX}(+BH_4, +L-arg). In Fe(II)NOS_{OX}(-BH_4, ± L-arg), we saw the (Fe(II) heme-O_2) decompose to produce mainly superoxide [8,22,23]. However, a new EPR signal appears in all three NOS isoforms when Fe(II)NOS_{OX}(+BH_4, −L-arg) reacts with O_2 [8]. This radical bears some resemblance to the BH_4 radical cation seen in normal turnover, but its EPR spectrum has more extensive wings,

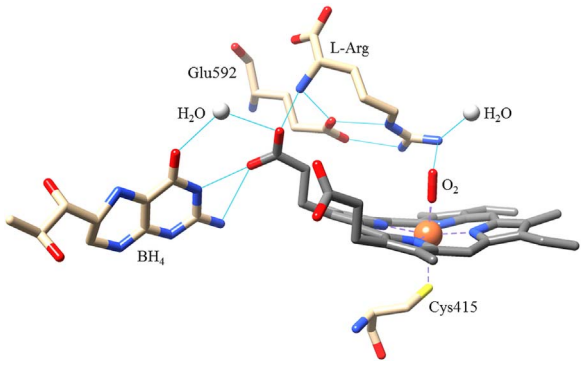


Fig. 1. Hydrogen-bonding among the "triad" of heme, BH_4 and L-arg. This network of bonds rigidifies the heme pocket scaffolding and regulates the heme spin-state changes caused by water ligation to the Fe(III). The O_2 bound to the heme is poised to attack the L-arg substrate. Adapted from the 1.85 Å resolution crystal structure of nNOSox, PDB: 2G6M.