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Comparison of wild type neuronal nitric oxide synthase and its Tyr588Phe mutant towards various L-arginine analogues

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ABSTRACT

Crystal structures of nitric oxide synthases (NOS) isoforms have shown the presence of a strongly conserved heme active-site residue, Tyr588 (numbering for rat neuronal NOS, nNOS). Preliminary biochemical studies have highlighted its importance in the binding and oxidation to NO of natural substrates L-Arg and N^{ω}-hydroxy-L-arginine (NOHA) and suggested its involvement in mechanism. We have used UV–visible and EPR spectroscopy to investigate the effects of the Tyr588 to Phe mutation on the heme-distal environment, on the binding of a large series of guanidines and *N*-hydroxyguanidines that differ from L-Arg and NOHA by the nature of their alkyl- or aryl-side chain, and on the abilities of wild type (WT) and mutant to oxidize these analogues with formation of NO. Our EPR experiments show that the heme environment of the Tyr588Phe mutant differs from that of WT nNOS. However, the addition of L-Arg to this mutant results in EPR spectra similar to that of WT nNOS. Tyr588Phe mutant binds L-Arg and NOHA with much weaker affinities than WT nNOS but both proteins bind non α-amino acid guanidines and *N*-hydroxyguanidines with close affinities. WT nNOS and mutant do not form NO from the tested guanidines but oxidize several *N*-hydroxyguanidines with formation of NO in almost identical rates. Our results show that the Tyr588Phe mutation induces structural modifications of the H-bonds network in the heme-distal site that alter the reactivity of the heme. They support recent spectroscopic and mechanistic studies that involve two distinct heme-based active species in the two steps of NOS mechanism.

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1. Introduction

In mammals, nitric oxide, NO, is produced by three isoforms of nitric oxide synthases (NOSs) that can be distinguished according to their initial cellular identifications, primary sequences and modes of regulation [1–3]. All three NOSs are heme-thiolate proteins that display significant structural and mechanistic similarities. NOSs have a homodimeric bidomain structure consisting of an NH_2-terminal oxygenase domain and a CO_2H-terminal reductase domain. The oxygenase domain binds the heme prosthetic group, substrate L-arginine (L-Arg) and cofactor (6R)-5,6,7,8-tetrahydro-L-biopterin (H4B) in close proximity to the heme, whereas the reductase domain contains binding sites for flavins, FMN and FAD, and for substrate NADPH. These two domains are bound by a calmodulin (CaM) binding sequence that triggers the electron flow from the reductase domain to the oxygenase domain as a function of free Ca^{2+} concentration [4,5]. All three NOSs catalyze the heme-dependent oxidation of L-Arg to L-citrulline and NO with intermediate formation of N^{ω}-hydroxy-L-arginine (NOHA) (Scheme 1) [6,7].

NO acts as a signalling agent in a huge range of physiological effects such as neurotransmission and vasodilatation. It is also involved in several pathologies associated to oxidative stress phenomena such as atherosclerosis and neurodegenerative diseases. Most of these diseases are linked to the concomitant formation of superoxide, hydrogen peroxide and peroxynitrite by NOSs [8–11]. Since the discovery that NOSs are important sources for superoxide and hydrogen peroxide, the elucidation of NOSs mechanism constitutes a major objective for the biomedical community [12–14]. The development of treatments for these diseases constitutes an important pharmacological challenge and many studies are undertaken to discover potent and selective inhibitors of NO-synthases [15,16].

In analogy with cytochromes P450, the following mechanism for NOS is presently proposed for NO formation from L-Arg. The first step of the NOS-dependent biosynthesis of NO (L-Arg hydroxylation to NOHA) would involve the formation of a ferric-peroxo complex upon one-electron reduction of the NOS–Fe^{II}–O_2 complex by H4B. Double protonation of the peroxo complex leads to an oxo-ferryl complex ($(Por^{\circ+})Fe^{IV}{=}O$) believed to be responsible for the hydroxylation of

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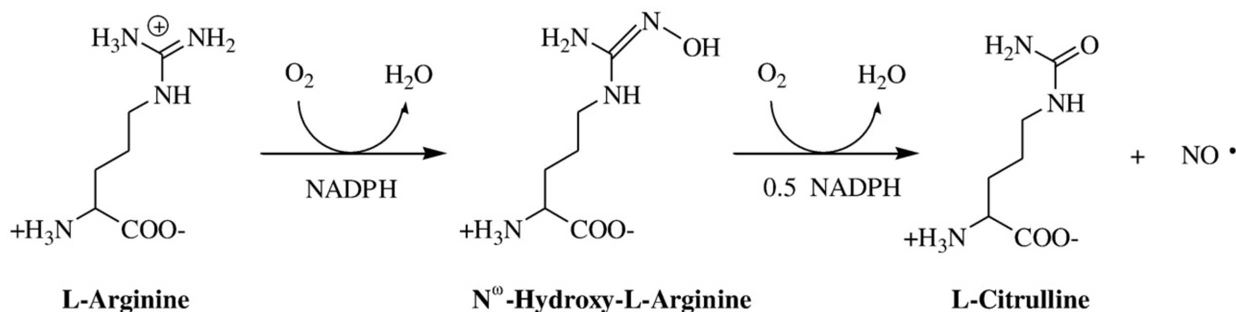

Scheme 1. The two steps of the reaction catalyzed by NOSs.

the guanidine moiety of L-Arg [3,17–21]. The second step of catalysis (NOHA oxidation to citrulline and NO) is believed to follow the same pathway up to the formation of the ferric-peroxo complex. Then, the catalytic sequence would involve either a direct nucleophilic attack of the hydroperoxo group [21] or of the peroxo species [22] on the hydroxyguanidinium carbon of NOHA. This reaction is followed by a rearrangement of the resulting tetrahedral complex with ultimate release of NO from an intermediate Fe^{III}–NO complex [23,24].

Crystal structures of the oxygenase domains of the three mammalian NOSs have shown very strong active-site conservation [25–29]. These structures identified key amino acid residues involved in the binding of substrates L-Arg and NOHA, and of H4B cofactor. They showed that these two substrates bind in an almost identical manner with their α-amino acid function as a key factor for binding at the NOSs active sites and a highly conserved crucial Glu residue (Glu592 in rat nNOS) H-bonded with the α-NH_2 and the guanidino- (or *N*-hydroxyguanidino) group of L-Arg (or NOHA) [29]. Furthermore, the crystallographic studies revealed a H-bond network involving L-Arg, diatomic ligands, and an active-site water molecule that could be involved in proton shuttling during catalysis [30,31]. They also identified H-bonds between the α-CO_2H group of L-Arg (or NOHA) and the OH-group of a highly conserved Tyr residue (Tyr588 in rat nNOS) to be of importance for recognition of both substrates at the heme active site (Fig. 1A). The Tyr588 aryl ring is close to the heme and to the aromatic rings of Trp587 and H4B, suggesting that this residue could play important roles in substrate binding and/or catalysis.

NOSs not only catalyze the oxidation of L-Arg and NOHA but also the oxidation of some non α-amino acid alkylguanidines (Gua) [32,33], and *N*-alkyl- and *N*-aryl-*N′*-hydroxyguanidines (NOHG) with formation of NO in reactions very similar to the oxidation of L-Arg and NOHA to citrulline and NO [33–36]. Different binding modes are reported for some aryl- and alkyl-NOHG in the substrate-binding pocket of NOSs [29,37]. In the case of *n*-butyl-NOHG, as observed with L-Arg and NOHA, the critical H-bonds established between the terminal NH_2 group and the δ-NH with Glu592 are conserved but the end of the butyl group curls towards Gln478 and Val567, away from the binding site for L-Arg or NOHA α-amino acid moiety (Fig. 1B) [29]. Different orientations are observed for close analogues. In the case of *i*-propyl-NOHG, the terminal NH_2 and NHOH groups of the *N*-hydroxyguanidine moiety always establish H-bond with Glu592 of nNOS and the methyl groups of the *i*-propyl moiety make van der Waals contacts with side chains of both Val567 and Phe584 but a clearly distinct orientation is observed for the OH group in comparison with *n*-butyl-NOHG [29]. In the case of 4-chlorophenyl-NOHG, the aryl-group is directed towards the heme-propionate of eNOS [37]. Anyway, the NOHG moiety of these small compounds remains in close proximity of the heme and reacts with the heme-active species to form NO [33–36].

In a preliminary study, we have studied the effects of Tyr588 mutations in nNOS on the binding affinity of L-Arg and NOHA and on the formation of NO from these two substrates. The Tyr588Phe mutation increased the dissociation constants for L-Arg and NOHA (8- and 2.8-fold, respectively), slightly reduced the formation of NO from L-Arg but stimulated the formation of NO from NOHA [38]. Similar effects were observed when the corresponding mutation was introduced into eNOS: the Tyr357Phe mutant of eNOS exhibited increased dissociation constants for L-Arg and NOHA (62- and 36-fold, respectively), a strongly reduced rate of NO formation from L-Arg but an almost identical rate of NO formation from NOHA [39]. The origins of these effects remained unclear. It was proposed that they could be linked to structural rearrangements in the distal site with changes in the substrate-binding

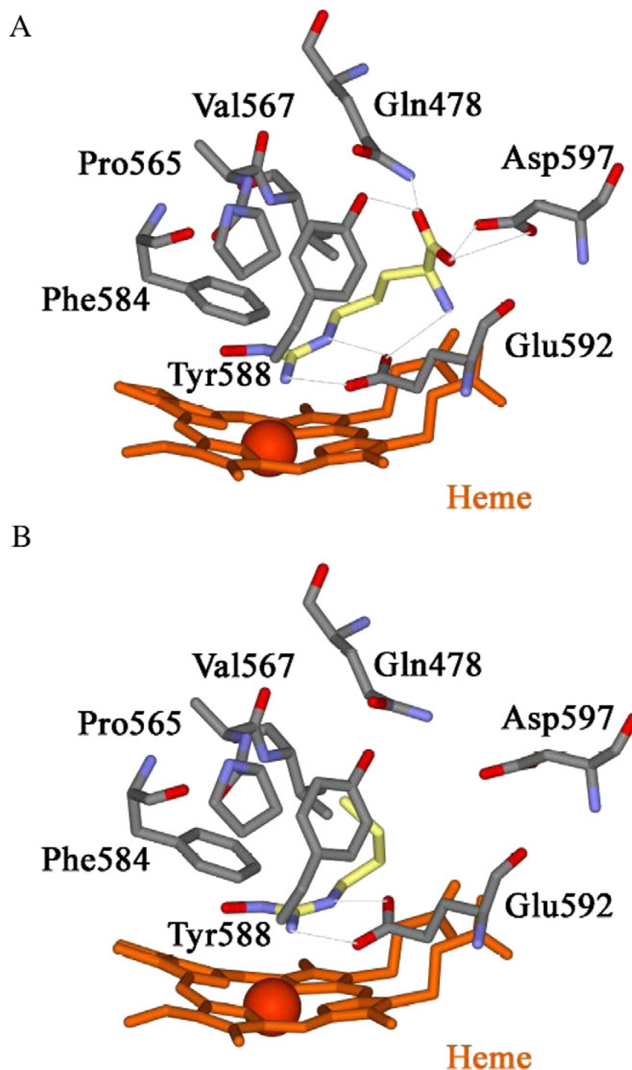

Fig. 1. (A) Structure of the active site of WT nNOS showing the main interactions between substrate NOHA and the residues close to the heme. Note the H-bonds (dotted lines) between the CO_2H-group of NOHA and the OH-group of Tyr588 (PDB access 1LZX). (B) Structure of the active site of WT nNOS in the presence of *n*-Butyl-NOHG (PDB access 1M00).

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