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Catalase activity of oxygenase domain of rat neuronal nitric oxide synthase. Evidence for product formation from L-arginine

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Abstract **Nitric oxide synthases (NOSs) catalyze the formation of nitric oxide from L-arginine. We purified the heme containing, tetrahydrobiopterin-free, oxygenase domain of rat neuronal nitric oxide synthase (nNOSox) overexpressed in *Escherichia coli*. We found catalase activity in nNOSox. This is significant because H_2O_2 may also be a product of nitric oxide synthases. We found H_2O_2 assisted product formation from *N*-hydroxy-L-arginine and even from L-arginine both in the presence and in absence of tetrahydrobiopterin. We propose how heme moiety of the oxygenase domain alone is sufficient to carry out both steps of the NOS catalysis.** **© 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Catalase; Nitric oxide synthase; Nitrite production; Tetrahydrobiopterin

1. Introduction

Mammals have at least three different isoforms of nitric oxide synthases (NOSs) all of which produce nitric oxide (NO) that has emerged as an important biomolecule involved in signal transduction, vasodilation and cytotoxicity [1–5]. Active as homodimer these enzymes catalyze two step oxidation of L-arginine (Arg) to *N*-hydroxy-L-arginine (NHA) and finally to NO and citrulline [6–8]. N-terminal oxygenase domain of this enzyme contains binding sites for heme iron protoporphyrin IX, tetrahydrobiopterin (H_4B) and Arg, and contains determinants for subunit dimeric interaction [8–10], and a C-terminal reductase domain of NOS binds calmodulin, FMN, FAD, and NADPH. The individual domains can fold and function independently and when mixed together show original activity [11,12]. The role of H_4B in NOS catalyzed reaction is not yet settled. No product was formed from Arg when H_4B-free NOS was tried as catalyst, accordingly it was suggested that H_4B plays the role in the first step of this NOS reaction to produce NHA [13]. In addition, no effect of catalase was found on the rate of citrulline production although H_2O_2 may be a product of the NOS reaction [13]. However, unlike pterin dependent hydroxylases NOS apparently does not release dihydrobiopterin after each turnover [14,15]. Here we report that nNOSox possesses a catalase activity. This enzyme also catalyzes product formation from Arg, in the H_2O_2 assisted reaction even without H_4B.

2. Materials and methods

2.1. Reagents

Clone of oxygenase domain of rat neuronal nitric oxide synthase, nNOSox (amino acid: 1–723), with a C-terminal hexa histidine tag, was a gift from Dr. Dennis J. Stuehr, Cleveland Clinic Foundation, Cleveland, USA. All other reagents and materials were obtained from Sigma or from sources previously reported [16,17].

2.2. Preparation and purification of nNOSox

Enzyme proteins were prepared as described previously [16,17]. Heme protein content was measured spectrophotometrically on a Hitachi-2001 spectrophotometer in the presence and absence of H_4B and Arg. Protein samples diluted in 40 mM MOPS, pH 7.6, containing 10% glycerol, 1 mM DTT, 10 μM H_4B and 0.4 mM imidazole to promote formation of fully low spin heme prior to titration. Spectra were recorded 30 min after each addition of Arg. Protein was estimated using protein assay kit (Bio-Rad, USA) with bovine serum albumin as standard. Purification was also checked by SDS–PAGE and judged to be more than 90% for nNOSox. Purified protein containing 1 mM DTT and 50% glycerol was kept in aliquots at −70°C until further use.

2.3. Dissociation of H_2O_2 at 240 nm

Dissociation of H_2O_2 (starting $A_{240} = 0.9$ diluted in 0.05 M phosphate buffer, pH 7.0) was measured with varying concentrations (60 nM to 2 μM) of nNOSox and also with varying H_2O_2 concentrations (1–26.4 mM) on 115 nM of nNOSox in the presence of 50-fold excess EDTA [19]. Catalase inhibitors like sodium azide and amitrazole were used to understand the biochemical nature of the reaction with 115 nM of nNOSox and 15.4 mM or 8 mM H_2O_2 respectively.

2.4. Measurement of molecular oxygen produced

Stock H_2O_2 (8.8 M) was diluted in 0.05 M phosphate buffer pH 7.0 and oxygen releasing activity was measured in 0.05 M phosphate buffer pH 7.0 with a fixed nNOSox concentration (115 nM) and H_2O_2 concentration (100 μM) by a Gilson 5/6 oxygraph. Final reaction volume was 1.8 ml [20].

2.5. Peroxidase activity measurement

Peroxidase activity was measured with 600 nM nNOSox as done for hemoglobin using citrate–phosphate buffer of pH 5.4 (0.05 M), 0.2% *O*-dianisidine and 17.6 mM H_2O_2 and also separately with 17.6 mM benzoyl peroxide [21].

2.6. Product formation from Arg and NHA

Hydrogen peroxide assisted product formation from Arg and NHA by nNOSox was assayed in 200 μl final volume. Assay mixture contains 40 mM MOPS, pH 7.6, 88 mM H_2O_2, 50 μg/ml bovine serum albumin and 0.5 mM DTT and varying concentrations of Arg or nNOSox protein. Assay was also performed in the presence of varying concentrations of *N*-nitro-Arg, an nNOS inhibitor. Product formation from NHA was done in the presence of 150 nM nNOSox protein and 88 mM H_2O_2. Reaction was started with H_2O_2 and incubated at 25°C

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Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; H_4B, (6*R*,6*S*)-2-amino-4-hydroxy-6-(*l*-erythro-1,2-hydroxypropyl)-5,6,7,8-tetrahydropterin; MOPS, 3-(*N*-morpholino) propanesulfonic acid; DTT, dithiothreitol; Arg, L-arginine; nNOSox, rat neuronal nitric oxide synthase oxygenase domain; NHA, *N*-hydroxy-L-arginine

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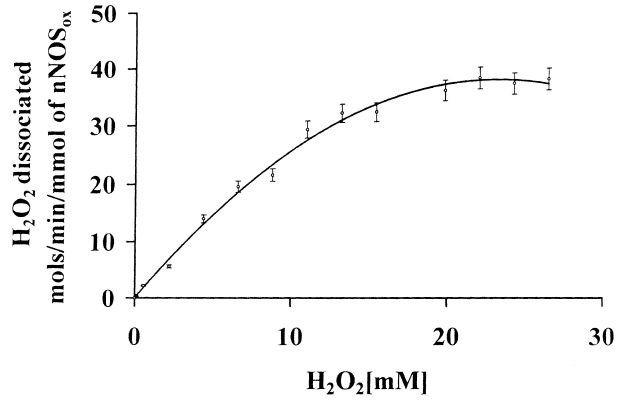

Fig. 1. Catalase activity of nNOSox. Stock H_2O_2 (8.8 M) was diluted in 0.05 M phosphate buffer pH 7.0 and hydrogen peroxide cleaving activity was measured at 240 nm in 0.05 M phosphate buffer pH 7.0 with a fixed nNOSox concentration (115 nM) in the presence of 50-fold excess of EDTA and varying H_2O_2 concentrations (0–26.4 mM). Final reaction volume was 300 μl. A classical Michaelis–Menten like curve was obtained. The data shown are representatives of replicate experiments of three separate enzyme preparations.

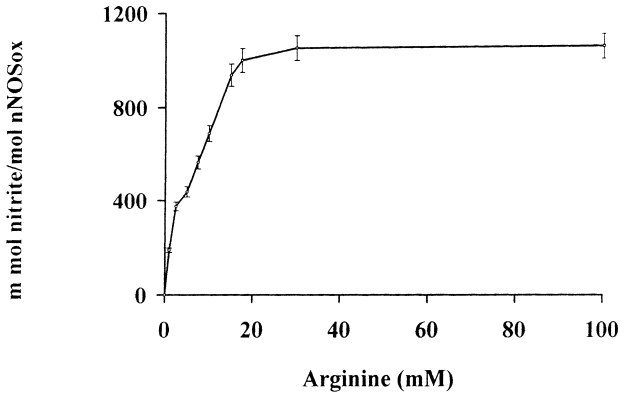

Fig. 3. H_2O_2 assisted product formation from Arg by H_4B-free nNOSox. Enzyme (6 μM) was incubated at 25°C with 88 mM H_2O_2, 50 μg/ml BSA, 0.5 mM DTT and Arg (0–100 mM) in 40 mM MOPS, pH 7.6 for 1 h and then the product formed was measured using Griess reagent. Final reaction volume was 200 μl. The experiment shown is representative of replicate experiments done at least six times with three separate enzyme preparations.

for 1 h. Product formed was quantitated with Griess reagent as mentioned earlier [16,18]. Product formation was also tried without nNOSox and in the presence of 12 μM hemoglobin (HbA_0).

3. Results

Recombinant protein of nNOSox was purified from extracts of *Escherichia coli* and quantitated spectrophotometrically using 10 μM H_4B and 3 mM Arg (data not shown). This nNOSox showed catalase activity with H_2O_2 with an apparent K_m of 7.35 mM (Fig. 1). This catalase activity of nNOSox was inhibited by sodium azide with an apparent IC_{50} of 305 μM and amitrazole with an apparent IC_{50} of 5.6 mM (not shown). Apparent turnover number is 6400 min^{-1}. Oxygen produced from dissociation of H_2O_2 measured in oxygraphic method (Fig. 2) highly corroborates with spectroscopically measured H_2O_2 dissociation (115 nm nNOSox and 100 μM H_2O_2 dissociates 0.34 mol/min/mmol nNOSox and 0.16 mol/min/mmol nNOSox oxygen produced).

The enzyme nNOSox also showed peroxidation activity with both H_2O_2 and benzoyl peroxide (data not shown).

Hydrogen peroxide assisted product formation from Arg was observed with H_4B-free nNOSox (Fig. 3). Product formation by nNOSox could be inhibited by *N*-nitro-L-arginine (Fig. 4). Product formation with H_4B (10 μM) increases about 60% product formation. The enzyme nNOSox is stable towards H_2O_2 for at least 1 h at 25°C (data not shown). Hemoglobin (12 μM), as HbA_0 prepared by gel filtration through Sephadex G-25 and then by ion exchange chromatography using Biorex-70 [22], did not produce measurable amount of product under the identical condition.

4. Discussion

We overexpressed oxygenase domain of rat neuronal nitric oxide synthase, nNOSox in *E. coli*. We found catalase activity with H_4B-free nNOSox in the absence of Arg (Fig. 1) which is inhibited by both sodium azide and amitrazole. This catalase activity is of mixed function and it is associated with peroxidation activity for both H_2O_2 and benzoyl peroxide. This is a newly described property of NOS. Ferric NOS could complex with H_2O_2 and in the absence of any other nucleophile acceptor like Arg/NHA reduce heme to ferrous state and produce molecular oxygen (Figs. 2 and 6). This model easily explains both the catalase activity of NOSs and also formation of peroxide by activated NOSs. Apparently NOS is equipped with catalase activity to protect itself from its own product, H_2O_2. Insensitivity of NOS towards addition of catalase from outside [14] now could be easily explained in the light of our findings. It is interesting to note that NOS distal heme pocket, primarily constructed from β-structure, differs considerably from the distal pockets of heme based peroxidases, catalases which are largely α-helical [23]. Superoxide radicals were

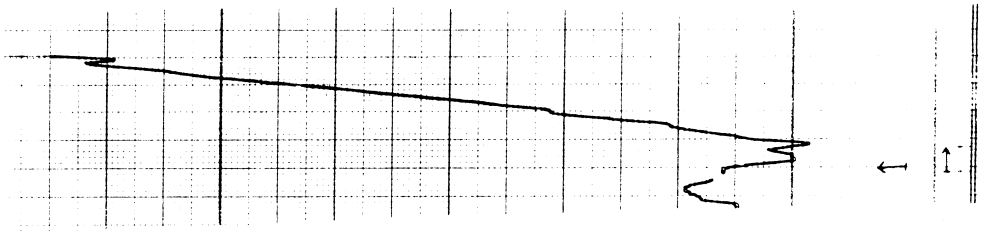

Fig. 2. Evolution of molecular oxygen by nNOSox from H_2O_2. Stocks were prepared as in Fig. 1 and oxygen releasing activity was measured in 0.05 M phosphate buffer pH 7.0 with a fixed enzyme concentration (115 nM) and H_2O_2 concentration (100 μM). Vertical arrow unit is equal to 1 nmol of oxygen produced and the horizontal arrow unit is equal to 90″ (one smallest unit = 20″). Final reaction volume was 1.8 ml. The data shown are representatives of replicate experiments of three separate enzyme preparations.

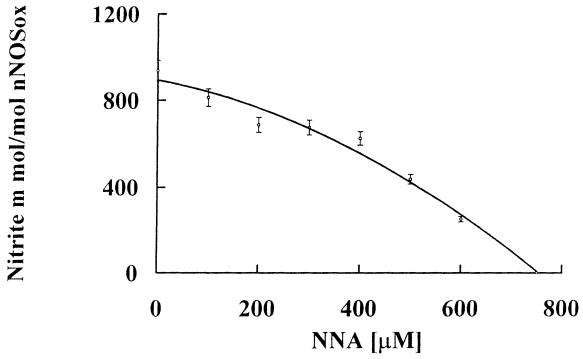

Fig. 4. *N*-Nitro-L-arginine inhibition of H_2O_2 assisted product formation from Arg by H_4B-free nNOSox. Enzyme (6 μM) was incubated at 25°C with 88 mM H_2O_2, 50 μg/ml BSA, 0.5 mM DTT, Arg (15 mM) and NNA (0–750 μM) in 40 mM MOPS, pH 7.6 for 1 h and then product formed was measured using Griess reagent. Final reaction volume was 300 μl. The experiment shown is representative of replicate experiments done at least six times with three separate enzyme preparations.

formed from oxygen by nNOSox ([24], and references therein). Using relatively lower concentrations of nNOSox (50–500 nM) no measurable product was formed from Arg, as also reported earlier [13,18]. It was claimed that H_4B directly takes part in the hydroxylation in the first step of NOS catalysis [13]. However in contrast to the previously characterized pterin dependent hydroxylases, NOS apparently does not release dihydrobiopterin after each turnover [14]. In aromatic amino acid hydroxylase, pterin plays hydroxylation reaction [25]. Measurable product was formed by nNOSox from Arg with

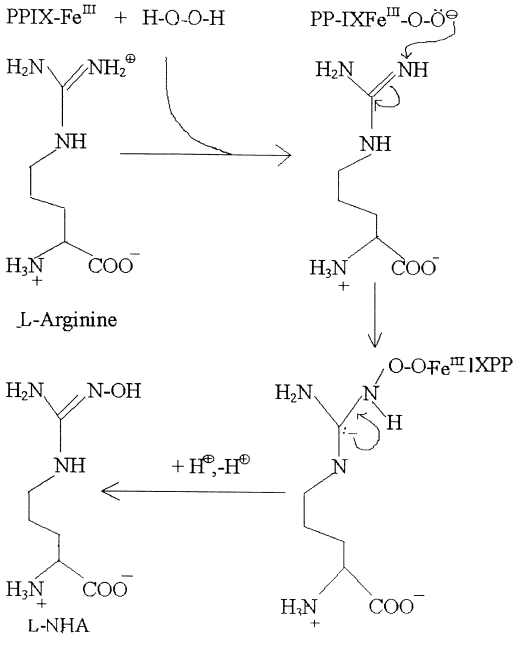

Fig. 5. Proposed mechanism for *N*-hydroxylation of Arg. This first step of N–O bond formation is much more difficult thermodynamically than the second step forming C–O bond as it has to go through a carbanion transition state. PPIX–Fe^{III}–O–O$^-$ once formed, these steps are highly probable. This is partly based on [13].

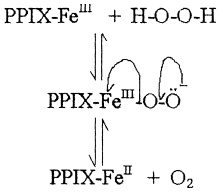

Fig. 6. Proposed mechanism for catalase activity of NOS. Ferric NOS will complex with H_2O_2 and in the absence of any other nucleophile acceptor like Arg/NHA reduce heme to ferrous state and produce molecular oxygen. PPIX–Fe^{III}–O–O$^-$ once formed, it is possible that Fe^{III} could be reduced to Fe^{II} by taking an electron with the evolution of molecular oxygen. As molecular oxygen leaves the reaction system, this reaction could be significant.

relatively high concentration of nNOSox (Fig. 3) but not by twice as much of other heme protein like hemoglobin. This product formation was inhibited with *N*-nitro-Arg (Fig. 4). The enzyme showed an apparent turnover number of 1/60 min^{-1} accordingly more enzymes were needed. This is complicated by the fact that all three reactants act on each other and accordingly we get a lower amount of product. Taken together our findings have great mechanistic implications for catalysis by NOS. Product could be formed from Arg by the H_4B-free nNOSox only with the help of an electron donor like H_2O_2 indicating heme alone is sufficient to carry out both the steps of the reaction. But a 3600 times lower activity [26] is suggestive of the role of reductase domain and H_4B in full length NOS catalysis. Of the two steps of the NOS catalysis, the first step is a hydroxylation reaction and NHA is formed from Arg. In the second step of the reaction, citrulline and NO are formed from NHA. Thermodynamically the first step of nitric oxide synthesis is more difficult because it involves N–O bond formation via a carbanion like transition state as opposed to the second step which involves C–O bond formation (Fig. 5). We propose that tetrahydrobiopterin and reductase domain somehow facilitate, by lowering the activation energy, the formation and/or stabilization of the carbanion like transition state. To our knowledge this is the first report of product formation from Arg by the oxygenase domain of any NOS even in the absence of H_4B. From crystal structure of the iNOS heme domain dimer, apparently H_4B is not involved in direct catalysis [23]. Heme alone is necessary and sufficient to form product from Arg.

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