Journal of Bioscience and Bioengineering
VOL. 118 No. 6, 616–621, 2014

www.elsevier.com/locate/jbiosc

Reduction of nitric oxide catalyzed by hydroxylamine oxidoreductase from an anammox bacterium

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Received 5 February 2014; accepted 27 May 2014
Available online 1 July 2014

The hydroxylamine oxidoreductase (HAO) from the anammox bacterium, *Candidatus* Kuenenia stuttgartiensis has been reported to catalyze the oxidation of hydroxylamine (NH_2OH) to nitric oxide (NO) by using bovine cytochrome *c* as an oxidant. In contrast, we investigated whether the HAO from anammox bacterium strain KSU-1 could catalyze the reduction of NO with reduced benzyl viologen (BV_{red}) and the NO-releasing reagent, NOC 7. The reduction proceeded, resulting in the formation of NH_2OH as a product. The oxidation rate of BV_{red} was proportional to the concentration of BV_{red} itself for a short period in each experiment, a situation that was termed quasi-steady state. The analyses of the states at various concentrations of HAO allowed us to determine the rate constant for the catalytic reaction, $(2.85 \pm 0.19) \times 10^5\ M^{-1}\ s^{-1}$, governing NO reduction by BV_{red} and HAO, which was comparable to that reported for the HAO from the ammonium oxidizer, *Nitrosomonas* with reduced methyl viologen. These results suggest that the anammox HAO functions to adjust anammox by inter-conversion of NO and NH_2OH depending on the redox potential of the physiological electron transfer protein in anammox bacteria.

[**Key words:** Anammox; Anaerobic ammonium oxidation; Hydroxylamine oxidoreductase; Nitric oxide; Hydroxylamine]

Hydroxylamine oxidoreductase (HAO) is a well-known homotrimeric protein composed of multi-heme subunits that catalyzes the oxidation of hydroxylamine (NH_2OH) to nitrite in ammonium oxidizing bacteria (AOB) such as *Nitrosomonas europaea*. *N. europaea* gains energy by aerobically oxidizing ammonium with oxygen, in a process during which HAO oxidizes the intermediate, NH_2OH, to nitrite (1). The molecular features of the purified HAO from *N. europaea* have been reported (2–6).

In recent years, it has become apparent that AOB such as *N. europaea*, which were once thought to be obligate aerobes, can in fact respire aerobically or anaerobically (6,7). During the transition from aerobic to anaerobic growth, the toxic intermediates NH_2OH and nitric oxide (NO) may accumulate as the O_2 supply diminishes. A kinetic study of the HAO from AOB using reduced methyl-viologen as the reductant (standard redox potential, −440 mV) was carried out and showed that HAO can catalyze two-step reductions of NO to NH_2OH, and NH_2OH to ammonium at substantial rates. The experiments were performed with NO generated *in situ* using the laser-photolysis method, and with analyses for initial reduction velocities of NO by HAO. According to the report, the rate constants governing each step were $(4.7 \pm 0.3) \times 10^5\ M^{-1}\ s^{-1}$ and $(2.06 \pm 0.04) \times 10^4\ M^{-1}\ s^{-1}$ per subunit, respectively (7,8).

Proteins similar to the HAO of AOB have been isolated and characterized from bacteria that catalyze anaerobic ammonium oxidation (anammox) (9) although their primary structures exhibit very low identities (<30%) to those of AOB (10,11). Anammox bacteria anaerobically oxidize ammonium with nitrite to gain energy, forming dinitrogen gas as shown in Eq. 1 (12,13).

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \quad (1)$$

A model for the mechanism of anammox was also proposed based on genes deduced from genome sequence analysis of the anammox bacterium *Candidatus* Kuenenia stuttgartiensis, and multi-heme proteins like HAO were proposed to function in the oxidation of the intermediate hydrazine (N_2H_4) to dinitrogen gas (13–15). We purified two kinds of HAO-like proteins that were expressed in a large quantity from the anammox bacterium strain KSU-1 (16), which belongs to a different genus from the *Candidatus* genus Kuenenia, and named the enzymes hydrazine-oxidizing enzyme (HZO) and HAO (10,11). We determined the kinetic properties of the HZO and the HAO for N_2H_4 and NH_2OH oxidation. HZO has a high affinity for hydrazine and activity with hydrazine (K_m, 5.5 μM; V_{max}, 6.2 μmol min^{-1} mg^{-1} protein) (11) compared to those of the HAOs from *Candidatus* Brocadia anammoxidans, *Candidatus* K. stuttgartiensis and strain KSU-1 (K_m, 25 μM; V_{max}, 0.54 μmol min^{-1} mg^{-1} protein) (Table S1). In addition, the HZO from strain KSU-1 is unable to oxidize hydroxylamine (NH_2OH) and the hydrazine-oxidizing activity is strongly inhibited by NH_2OH (K_i, 2.4 μM). However, the HAO showed comparably high oxidation rates with another substrate, NH_2OH (10), although the model does not explain how NH_2OH is formed in anammox bacterial cells (Table S1).

Recently, the HAOs from the anammox bacterium *Candidatus* K. stuttgartiensis and *N. europaea*, were re-evaluated (17). The results

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1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2014.05.018

indicated that the former enzyme catalyzes a three-electron oxidation of NH_2OH to NO using bovine cytochrome *c* as an oxidant, whereas the latter catalyzes a four-electron oxidation. The results supported the idea that HAOs in the anammox bacteria function physiologically to diminish the inhibition of N_2H_4 oxidation by decreasing the concentration of NH_2OH (10,17).

$$NH_2OH + n \text{ cytochrome } c \rightarrow \rightarrow \rightarrow NO \rightarrow NO_2^- \quad (n: 3 \text{ or } 4;\ \rightarrow: \text{one electron oxidation}) \qquad (2)$$

Considering the bidirectional reaction catalyzed by the HAO of AOB, and as it is reported that NO is synthesized as an intermediate either by a cytochrome cd_1-type nitrite reductase (Nir), or a Cu-type Nir in anammox bacteria (13,18), we proposed that the anammox HAO could catalyze the inter-conversion of NO and NH_2OH. To confirm the reduction of NO, we investigated whether the KSU-1 HAO could reduce NO to NH_2OH in the presence of reduced benzyl viologen (standard redox potential, −358 mV), and determined the rate constants governing the reaction in this study.

MATERIALS AND METHODS

Enzymes and reagents The HAO and HZO enzymes were purified from sludge mainly containing strain KSU-1 according to our previous reports (10,11). 1-Hydroxy-2-oxo-3-(*N*-methyl-3-amino-propyl)-3-methyl-1-triazene (NOC 7) was purchased from Chemical Dojin Corp (Kumamoto, Japan). NOC 7 is stable in alkaline pH, but decomposes gradually at neutral pH, releasing NO (19,20). One mol of NOC 7 releases 2 mol of NO as shown in Fig. S1. Its half-life is 5 min in PBS pH 7.4 at 37°C. Sodium hydrosulfite and 1,1′-dibenzyl-4,4′-bipyridinium dichloride hydrate (benzyl viologen) were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Argon gas used in experiments described below was 99.999% pure.

Experimental procedures The reaction mixture (1.5 mL) for reduction of nitrite or NO by HAO or HZO, which contained potassium phosphate buffer, pH 7.0 (final concentration 45 mM), nitrite (final concentration 0.2 mM), purified HAO or HZO, and fully oxidized benzyl viologen (BV_{ox}) (final 0.3 mM), was sealed in a cuvette with a butyl rubber cap. Then, argon gas was blown through a syringe into the cuvette to purge oxygen. Sodium hydrosulfite was added with a syringe to transform two thirds of the BV_{ox} to the reduced form, BV_{red}, and the oxidation of BV_{red} (absorbance at 550 nm, extinction coefficient: 10.4 mM^{-1} cm^{-1}) at 35°C was followed with a spectrophotometer (MPS-2400; Shimadzu Corporation, Kyoto, Japan). NOC 7 was dissolved to a final concentration of 50 mM in 100 mM of NaOH solution that had been sparged with argon gas. Using a syringe, 6 μL of NOC 7 solution (final concentration 0.2 mM) was added into the cuvette, which was immediately removed from the spectrophotometer, inverted to mix the solution, and returned to the fixed location in the spectrophotometer. The absorbance of BV_{red} was continuously recorded in a Microsoft Excel file during these operations. The concentrations of NH_2OH formed were assayed after the reaction.

Chemical oxidation of BV_{red} with NO was separately analyzed. To prepare NO-saturated buffer, authentic NO gas passed through 1 M NaOH was blown into 0.2 M potassium phosphate buffer, pH 7.0, from which oxygen had been previously purged with argon gas [approximately 1.9 mM NO estimated based on the solubility in pure water at 20°C (21)]. The reaction mixture contained 100 μM BV_{red} and 200 μM BV_{ox} before the reaction was initiated as described above. The reaction was started with the injection of an aliquot (20 μL) of NO-saturated buffer into the reaction mixture (1.5 mL) without the enzyme through rubber cap, and the oxidation of BV_{red} was measured at 550 nm. These experiments were performed twice.

Data processing All data were recorded in a Microsoft Excel file. The decrease in the concentration of BV_{red} for any 0.2 s was converted into a rate per minute and the values obtained were plotted against the concentration of BV_{red}.

Other analytical methods Protein concentrations were determined with the BCA protein assay kit (Pierce, Rockford, IL, USA), using bovine serum albumin as the standard (22). The HAO concentration in μM was calculated using the protein concentration and the molecular mass of a trimeric protein because we obtained data regarding the steric structure of KSU-1 HAO, which indicated the protein was a trimer (data not shown). Hydroxylamine concentrations were determined using 8-quinolinol as previously described (23). Ammonium concentrations were assayed with an ammonium test kit (Wako Pure Chemical Industries, Ltd., Osaka).

RESULTS

Substrates for strain KSU-1 HAO The ability of HAO and HZO to reduce nitrite, NO or NH_2OH as substrates in the presence of BV_{red} was investigated. When sodium nitrite or NH_2OH was added (0.2 mM final concentration), neither HAO nor HZO reduced the substrates with BV_{red}. When 0.2 mM of the NO-releasing reagent, NOC 7, was used, HAO (110 nM) accelerated the rate of BV_{red} oxidation compared to the control reaction without HAO (Fig. 1). In contrast, HZO did not enhance the oxidation (data not shown). In the reaction catalyzed by HAO, NH_2OH was formed as a product, but ammonium was not. The concentration of NH_2OH was 40 ± 6.7 μM ($n = 3$) at the end of the reaction. This indicates that the HAO could catalyze the reduction of NO to NH_2OH. The rate of NH_2OH formation with varying concentrations of HAO was also measured, as well as the length of time required to completely oxidize BV_{red}. The results (summarized in Table 1) show that the elapsed time became shorter and the rate of NH_2OH formation increased as the concentration of HAO increased. These results support a 3-electron reduction of nitric oxide to hydroxylamine catalyzed by HAO.

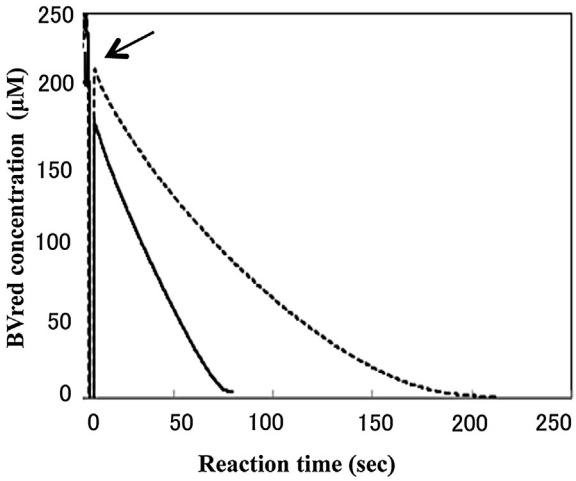

FIG. 1. Oxidation of BV_{red} by NO released from NOC 7. The solid line indicates the reaction of BVred and NO released from NOC 7 (final concentration of 200 μM) in the presence of HAO (final concentration of 110 nM). The dashed line represents the control reaction without HAO. The arrow shows the time when NOC 7 was added to each reaction mixture.

Analysis of the BV_{red} oxidation rates The rates (the decreasing rates) of BV_{red} oxidation were calculated at 0.2-s intervals by using the Microsoft Excel data obtained in the experiments shown in Fig. 1, and plotted against the corresponding concentrations of BV_{red} (Fig. 2).

The chemical reaction of BV_{red} and NO without catalyst was shown in Fig. 2A. These results demonstrate that the decreasing rates of BV_{red} correlate linearly with the concentrations of BV_{red} in the range between approximately 30 μM and 110 μM, suggesting that sequentially released NO balanced with the NO consumed;

TABLE 1. The elapsed time and the amounts of hydroxylamine formed for the concentration of HAO added.

HAO (μM)	Elapsed time (min)	Hydroxylamine formed (μM)	Reducing equivalent calculated by hydroxylamine formed (μeq)	*C* value multiplied by the elapsed time (μM)
0	3.35 ± 0.10	~0	~0	–
0.05	1.67 ± 0.10	42.7 ± 7.0	128 ± 21	70.8
0.11	1.34 ± 0.09	43.5 ± 7.5	131 ± 23	56.8
0.16	1.10 ± 0.05	54.3 ± 8.0	163 ± 24	46.6
0.21	0.76 ± 0.05	50.5 ± 7.5	152 ± 23	32.2
0.27	0.89 ± 0.06	55.7 ± 0.8	167 ± 2.4	37.7

$n = 3$: decrease of BV_{red} was monitored by spectrophotometer. Hydroxylamine formed was measured after the reaction by colorimetric determination. Oxidized BV_{red} was 194 ± 5.9 μM. *C* value used was that in Table 2.