Nitric Oxide 31 (2013) 38–47

Contents lists available at SciVerse ScienceDirect

Nitric Oxide

journal homepage: www.elsevier.com/locate/yniox

Nitroxyl accelerates the oxidation of oxyhemoglobin by nitrite

Landon Bellavia [a], Jenna F. DuMond [b], Andreas Perlegas [a], S. Bruce King [b], Daniel B. Kim-Shapiro [a,*]

[a] Department of Physics, Wake Forest University, Winston-Salem, NC 27109, USA
[b] Department of Chemistry, Wake Forest University, Winston-Salem, NC 27109, USA

ARTICLE INFO

Article history:
Received 31 August 2012
Revised 19 March 2013
Available online 30 March 2013

Keywords:
Hemoglobin
Kinetics
Nitrite
Nitroxyl
Therapeutics

ABSTRACT

Angeli's salt ($Na_2N_2O_3$) decomposes into nitroxyl (HNO) and nitrite (NO_2^-), compounds of physiological and therapeutic interest for their impact on biological signaling both through nitric oxide and nitric oxide independent pathways. Both nitrite and HNO oxidize oxygenated hemoglobin to methemoglobin. Earlier work has shown that HNO catalyzes the reduction of nitrite by deoxygenated hemoglobin. In this work, we have shown that HNO accelerates the oxidation of oxygenated hemoglobin by NO_2^-. We have demonstrated this HNO mediated acceleration of the nitrite/oxygenated hemoglobin reaction with oxygenated hemoglobin being in excess to HNO and nitrite (as would be found under physiological conditions) by monitoring the formation of methemoglobin in the presence of Angeli's salt with and without added NO_2^-. In addition, this acceleration has been demonstrated using the HNO donor 4-nitrosotetrahydro-2*H*-pyran-4-yl pivalate, a water-soluble acyloxy nitroso compound that does not release NO_2^- but generates HNO in the presence of esterase. This HNO donor was used both with and without NO_2^- and acceleration of the NO_2^- induced formation of methemoglobin was observed. We found that the acceleration was not substantially affected by catalase, superoxide dismutase, c-PTIO, or IHP, suggesting that it is not due to formation of extramolecular peroxide, NO_2 or H_2O_2, or to modulation of allosteric properties. In addition, we found that the acceleration is not likely to be related to HNO binding to free reduced hemoglobin, as we found HNO binding to reduced hemoglobin to be much weaker than has previously been proposed. We suggest that the mechanism of the acceleration involves local propagation of autocatalysis in the nitrite-oxygenated Hb reaction. This acceleration of the nitrite oxyhemoglobin reaction could affect studies aimed at understanding physiological roles of HNO and perhaps nitrite and use of these agents in therapeutics such as hemolytic anemias, heart failure, and ischemia reperfusion injury.

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Introduction

Nitroxyl (HNO) is one-electron reduced from nitric oxide (NO) and is associated with several biochemical processes that, though sometimes resembling processes involving NO, possess distinct mechanisms and pathways[1–13]. HNO has been considered as the basis of a therapeutic strategy in cardiac systems associated with heart failure[14–21], in mitochondrial regulation [13], and in other pharmacological and biological signaling contexts both in vitro and in vivo [22–28]. Thus, Angeli's salt ($Na_2N_2O_3$, sodium α-oxyhyponitrite, AS) or other sources of nitroxyl (HNO) can potentially function as therapeutics for numerous conditions. The breakdown of Angeli's salt into HNO and nitrite has been studied for over a century [29–35].

Nitrite reacts with oxygenated hemoglobin (oxyHb) to form methemoglobin (metHb) [36,37], and the reaction becomes very efficient at high nitrite concentrations, where the reaction becomes autocatalytic [38–44]. Interest in nitrite has been increasing lately due to its emerging role as a vasodilator and source of bioavailable nitric oxide [45–48]. Nitrite has been shown to be a signaling molecule [49] with cytoprotective applications against ischemia–reperfusion injury [50,51].

Angeli's salt decomposes under physiological conditions with a first order rate constant of $k = 6 \times 10^{-4}\ s^{-1}$ to yield HNO and NO_2^- [32]. Early work [52] confirmed the stoichiometry of the reaction of HNO with oxyhemoglobin to form metHb and nitrate as

$$HNO + 2[HbO_2]^{2+} \rightarrow 2[Hb]^{3+} + NO_3^- + HO_2^- \quad (1)$$

through a proposed two-step mechanism of oxyHb oxidation by HNO to for m NO, followed by NO oxidation of a second oxyHb to form nitrate (Eqs. (2) and (3)).

$$HNO + [HbO_2]^{2+} \rightarrow 2[Hb]^{3+} + NO + HO_2^- \quad (2)$$

Abbreviations: NO, nitric oxide; HNO, nitroxyl; AS, Angeli's salt; Hb, hemoglobin; oxyHb or $[HbO_2]^{2+}$, oxygenated hemoglobin; deoxyHb, deoxygenated hemoglobin; metHb or $[Hb]^{3+}$, methemoglobin; PLE, pig liver esterase.

* Corresponding author. Fax: +1 336 758 6142.
E-mail address: shapiro@wfu.edu (D.B. Kim-Shapiro).

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http://dx.doi.org/10.1016/j.niox.2013.03.006

$$NO + [HbO_2]^{2+} \rightarrow [Hb]^{3+} + NO_3^- \quad (3)$$

Peroxide formed in Eq. (2) would be expected to rapidly form hydrogen peroxide at neutral pH. It should be noted that there has been debate regarding the pathway described in (Eq. (2)), and alternative pathways have been proposed [53]. The rate constant for (Eq. (2)) is likely to be on the order of $10^7\ M^{-1}\ s^{-1}$, the rate reported for the same reaction with oxymyoglobin [12]. The rate of (Eq. (3)) is somewhat faster ($k = 5–8 \times 10^7\ M^{-1}\ s^{-1}$) [54–56].

The overall reaction of nitrite with oxyHb can be described by (Eq. (4)).

$$4[HbO_2]^{2+} + 4NO_2^- + 4H^+ \rightarrow 4[Hb]^{3+} + 4NO_3^- + O_2 + 2H_2O \quad (4)$$

This reaction was first studied in 1868 by Arthur Gamgee [36] and has since been studied by many others [40,44,55,57–63]. The reaction progresses slowly at low nitrite concentrations ($k = 0.21–0.33\ M^{-1}\ s^{-1}$), but becomes autocatalytic at nitrite concentrations that are high relative to the oxyHb concentration.

Recent kinetic models by Keszler and coworkers [60] support a mechanism by which autocatalysis is initiated in a multi-step process. This scheme postulates the addition of nitrite to heme-bound oxygen to form a ferrous-peroxynitrate intermediate (Eq. (5)), which oxidizes nitrite to form nitrate and a ferrous-peroxynitrite intermediate (Eq. (6)). This proposed ferrous-peroxynitrite complex would then be reduced to nitric oxide and peroxide (Eq. (7)), and the resulting NO would rapidly oxidize a second oxyHb (Eq. (3)).

$$[HbO_2]^{2+} + NO_2^- \rightarrow [HbO_2NO_2]^+ \quad (5)$$

$$[HbO_2NO_2]^+ + NO_2^- \rightarrow [HbOONO]^+ + NO_3^- \quad (6)$$

$$[HbOONO]^+ + 2H^+ \rightarrow [Hb]^{3+} + NO + H_2O_2 \quad (7)$$

Allowing the reaction to progress slowly according to this scheme, peroxide would be expected to decompose to water and molecular oxygen, yielding a net reaction equivalent to (Eq. (4)). However, at sufficiently high concentrations of metHb and peroxide, these components react to form a ferryl hemoglobin radical (Eq. (8)), and it is this radical that is believed to initiate propagation of autocatalysis [44,62]. Under the current model, the ferryl hemoglobin radical reacts with nitrite to form NO_2 radical and ferryl hemoglobin (Eq. (9)), which reacts with an additional nitrite to form metHb and another NO_2 radical (Eq. (10)). NO_2 radicals formed in this process can react with oxyHb to form a peroxynitrate adduct (Eq. (11)), which decomposes to the ferryl hemoglobin radical and nitrate (Eq. (12)). Thus, the autocatalytic mechanism is propagated by the NO_2 radical and the cycling of ferryl and ferryl radical hemoglobins.

$$[Hb]^{3+} + H_2O_2 \rightarrow [^{\cdot}Hb = O]^{2+} + H_2O + H^+ \quad (8)$$

$$[^{\cdot}Hb = O]^{2+} + NO_2^- \rightarrow [Hb = O]^{2+} + {}^{\cdot}NO_2 \quad (9)$$

$$[Hb = O]^{2+} + NO_2^- \rightarrow [Hb]^{3+} + {}^{\cdot}NO_2 \quad (10)$$

$$[HbO_2]^{2+} + {}^{\cdot}NO_2 \rightarrow [HbOONO_2]^{2+} \quad (11)$$

$$[HbOONO_2]^{2+} + NO_2^- \rightarrow [^{\cdot}Hb = O]^{2+} + NO_3^- + H^+ \quad (12)$$

At low concentrations of nitrite, such as those used in our experiments, autocatalysis is not expected to occur, and the oxidation of hemoglobin by nitrite is predicted to progress according to (Eqs. (5)–(7) and (Eq. (3)).

In addition to oxidizing oxyHb, nitrite has also been shown to react with deoxyHb to form metHb and iron-nitrosyl hemoglobin [42,64,65]. Doyle and coworkers reported observing catalysis of the nitrite reaction with deoxyhemoglobin by HNO, but no such catalysis was reported for the reaction of nitrite with oxyHb [52]. In this paper, we show that HNO also accelerates the reaction of nitrite with oxyHb. We demonstrate this phenomenon using AS and using the newly developed HNO donor 4-nitrosotetrahydro-2*H*-pyran-4-yl pivalate [66] belonging to the recently described family of acyloxy nitroso compounds that yield HNO upon hydrolysis [67,68]. Importantly, these experiments have been performed with oxyHb in excess to both nitrite and HNO, as would be the case under physiological conditions.

Materials and methods

Reagents

Angeli's salt, DEA NONOate, and carboxy-PTIO (c-PTIO) were purchased from Caymen Chemical. Superoxide dismutase, diethylene triamine pentaacetic acid (DTPA), and phytic acid were purchased from Sigma Aldrich. Other chemicals and supplies were purchased through Fisher Scientific. Packed red blood cells used in the preparation of hemoglobin solution were purchased from Interstate Blood Bank (Memphis, TN, USA).

Hb was purified as described previously [69,70]. Red blood cells were washed in pH 7.4 PBS and lysed by dilution with distilled deionized water. The membranes were spun out by centrifugation at 17,000*g*. and the Hb was dialyzed against distilled deionized water and PBS. The Hb was pelleted in liquid nitrogen and stored at −80 °C for future use.

Angeli's salt and DEA NONOate stock solutions were prepared in 10 mM NaOH. The concentration of each stock solution was confirmed by absorbance at 250 nm, using an extinction coefficient (ε) of $8\ mM^{-1}\ cm^{-1}$ for Angeli's salt and $9\ mM^{-1}\ cm^{-1}$ for DEA NONOate [71]. Stock solutions of 10 mM $NaNO_2$ were also prepared in 0.01 M NaOH, with the concentration of $NaNO_2$ being determined by mass. Stock solutions of 10 mM c-PTIO were prepared in phosphate buffered saline, with the concentration of c-PTIO being determined by mass. Stock solutions of 10 mM inositol hexaphosphate (IHP) were prepared from phytic acid by titration with sodium hydroxide to pH 7.3.

4-nitrosotetrahydro-2*H*-pyran-4-yl pivalate was synthesized as described previously [66]. Esterase from porcine liver (pig liver esterase, PLE) was purchased from Sigma–Aldrich.

Spectroscopy

Angeli's salt, DEA NONOate, and initial oxyHb concentrations were verified on a Cary 50 bio-spectrometer (Varian, Inc.). Reactions involving Hb were monitored using time-resolved spectroscopy on a Cary 100 bio-spectrometer (Varian, Inc.) with a temperature controller set to maintain sample temperatures at 37 °C and a six-cell sample changer that facilitated scanning of up to six samples simultaneously under the same conditions.

Hemoglobin reactions were analyzed by spectral deconvolution using a least-squares fit to known basis spectra (Fig. 1A). A sample spectrum and the corresponding fit are shown in Fig. 1B. Of the species included in the basis spectra, oxyHb and metHb always accounted for a sum total amount >97% of the Hb species present. Data reported is for metHb levels, and remaining Hb is almost exclusively oxyHb, with occasional trace amounts (<2.5%) of deoxyHb found as the reactions progressed.

Nitrite analysis

Nitrite levels were assessed using a Sievers Nitric Oxide Analyzer (NOA) (GE Instruments) according to standard procedures for a NaI assay provided by the manufacturer. Nitrite levels

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