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ScienceDirect
Biochemical and Biophysical Research Communications 360 (2007) 146–148
BBRC
www.elsevier.com/locate/ybbrc

The glutathione thiyl radical does not react with nitrogen monoxide

Dustin Hofstetter, Thomas Nauser, Willem H. Koppenol *

Laboratory for Inorganic Chemistry, Department of Chemistry and Applied Bioscience, ETH Zurich, Wolfgang-Pauli-Strasse 10, CH-8093 Zurich, Switzerland

Received 30 May 2007
Available online 11 June 2007

Abstract

Laser flash photolysis experiments shows that the rate constant for the reaction of the glutathione thiyl radical with nitrogen monoxide to give *S*-nitrosoglutathione is lower than $2.8 \pm 0.6 \times 10^7\ M^{-1}\ s^{-1}$. The conversion of the thiyl radical to its carbon-centred form at $10^3\ s^{-1}$ exceeds the formation of *S*-nitrosoglutathione when physiological concentrations of nitrogen monoxide are taken into account.

Keywords: *S*-nitrosoglutathione; Nitrogen monoxide; Thiyl radical; Laser flash photolysis

Nitration and nitrosation of bioactive molecules has gained importance ever since the biological role of nitrogen monoxide was discovered in 1987 [1,2]. It has become associated with oxidative stress and disease [3], subsequently, *S*-nitrosated amino acids, peptides and, proteins including cysteine, glutathione, or serum albumin have been detected, and their biosyntheses were tentatively attributed to reaction with nitrogen monoxide [4–7]. The crucial step in *S*-nitrosothiol biosynthesis is believed to be the reaction of nitrogen monoxide with a thiyl radical [6,7]:

$$RS^{\cdot} + NO^{\cdot} \rightarrow RSNO \quad (1)$$

To date, only an estimate exists for the rate constant for Eq. (1), namely $k_1^{est} \approx 10^9\ M^{-1}\ s^{-1}$ [6,8], and this value has been used in various models [6–8].

One particularly abundant thiol in the cell is glutathione [9]. Its *S*-nitrosated derivative is also relatively abundant in vivo and does not decompose in aqueous solution at room temperature [10,11]. Because of its facile synthesis, *S*-nitrosoglutathione has been widely studied.

The concentration of nitrogen monoxide in the physiological environment is approximately 10^{-7} M [13], which, combined with k_1^{est}, gives a first-order rate constant of $k_1'^{est} \approx 10^2\ s^{-1}$. However, a competing reaction, namely the intramolecular radical rearrangement [12,14,15] must also be considered:

$$GS^{\cdot} \rightleftarrows {}^{\cdot}GS \quad (2)$$

In this hydroxide-dependent rearrangement, the thiyl radical abstracts a H atom from an accessible tertiary carbon proximal to a carboxylate group. The equilibrium of Eq. (2) lies to the right, and the rate constant k_2 is approximately $10^3\ s^{-1}$ at physiological pH [14,15]. A comparison of $k_1'^{est}$ and k_2 shows that a glutathione thiyl radical is more likely to rearrange to glutathione carbon-centred radical than to react with nitrogen monoxide.

In this work, we derive an upper limit of $(2.8 \pm 0.6) \times 10^7\ M^{-1}\ s^{-1}$ for k_1, much lower than the estimate of $10^9\ M^{-1}\ s^{-1}$ and much lower than expected for a radical reaction.

Materials and methods

Reduced and oxidised glutathione (both 98%) were obtained from ACROS Organics (Geel, Belgium), and *S*-nitrosoglutathione was prepared according to the literature [16]. Sodium dihydrogen phosphate–water (1/2) and sodium hydrogen phosphate–water (1/12) were from Fluka (Buchs,

Abbreviations: GSNO, *S*-nitrosoglutathione; GS$^{\cdot}$, glutathione thiyl radical; $^{\cdot}$GS, Glutathione carbon-centred radical; RSNO, unspecified *S*-nitrosothiol; RS$^{\cdot}$, unspecified thiyl radical.

* Corresponding author. Fax: +41 446321090.
E-mail address: koppenol@inorg.chem.ethz.ch (W.H. Koppenol).

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doi:10.1016/j.bbrc.2007.06.008

Switzerland). 2-Methyl-2-propanol (purum) was recrystallised. Argon (5.0, 99.999%) and nitrogen monoxide (2.0, 99.5%) gases were from Linde (Höllriegelskreuth, Germany). Milliporte Milli-Q purified water was used throughout.

Solutions of glutathione and its derivatives were freshly prepared in 100 mM 2-methyl-2-propanol solution in sodium phosphate buffer at pH 7.3. The solutions, contained in gas-tight fluorescence cells were evacuated to boiling and subsequently filled it with argon twice, then, after a further evacuation, argon or nitrogen monoxide gas was added to saturation. The resulting concentration of nitrogen monoxide is 1.8×10^{-3} M [17,18].

Laser flash photolysis was conducted with an Applied Photophysics LKS 50 instrument fitted with a Brilliant B Nd:YAG Laser providing a 4th harmonic at 266 nm with 5 ns pulses. Single pulse energies were kept lower than 20 mJ to avoid water photolysis [19]. The photolysis of oxidised glutathione yields two glutathione thiyl radicals [20], and that of *S*-nitrosoglutathione yields glutathione thiyl radical and nitrogen monoxide [12]. The reactions were followed at 330 nm. Digital filters were used to reduce high frequency noise.

Curve fitting was carried out with *Kaleidagraph* software, and kinetics constants were estimated with a pseudo-first-order model, given that $[GS^{\bullet}] \ll [NO^{\bullet}]$.

Results

After flashing with a laser pulse, the absorption of *S*-nitrosoglutathione ($\varepsilon_{330\,nm} = 850\ cm^{-1}\ M^{-1}$) in an argon-saturated solution bleaches immediately (Fig. 1), but this absorption does not recover to the original level within 10^{-4} s. This shows that *S*-nitrosoglutathione undergoes homolysis to form nitrogen monoxide and the glutathione thiyl radical ($\varepsilon_{330\,nm} = 580\ cm^{-1}\ M^{-1}$ [21]), but does not regenerate. The same experiment carried out with nitrogen monoxide instead of argon shows the same outcome. Even under conditions of excess nitrogen monoxide (1.8×10^{-3} M), the formation of *S*-nitrosoglutathione is not observed.

When 2×10^{-3} M oxidised glutathione ($\varepsilon_{330\,nm} < 1\ cm^{-1}\ M^{-1}$) is irradiated in argon-saturated solution, the immediate formation and decay of the glutathione thiyl radical (Fig. 2A) is observed. The subsequent increase in absorption can be assigned to formation of a carbon-centred radical species [14,15]. In the presence of nitrogen monoxide, the observed absorption change is larger. At the absorption maximum of *S*-nitrosoglutathione, 330 nm, the difference spectrum (Fig. 2B) reveals that a second process occurs in the nitrogen monoxide saturated solution. If we assume that this difference is caused by reaction (1b), we obtain $k_{1b} \leqslant 2.8 \pm 0.6 \times 10^7\ M^{-1}\ s^{-1}$.

Discussion

The results of our study do not allow us to speculate about the biosynthesis pathway of *S*-nitrosoglutathione. However, our results do rule out a direct radical mechanism. The derived upper limit of $2.8 \pm 0.6 \times 10^7\ M^{-1}\ s^{-1}$ for k_{1b} is much lower than expected for a radical mechanism, and the first-order rate constant in the presence of a physiological nitrogen monoxide concentration would be $2.8 \pm 0.6\ s^{-1}$. The rate of Eq. (2) is about 300 times higher than that of Eq. (1), even in the presence of 1.8×10^{-3} M nitrogen monoxide, and, for that reason, Eq. (1) does not likely take place under physiological conditions. This conclusion, however, holds for homogeneous solution only; one could imagine that Eq. (1) could proceed within the active site of an enzyme. Given that the number of tertiary carbons in a protein that carry an α-carboxylate group is large, a glutathione thiyl radical is more likely to abstract a hydrogen from protein than to react with nitrogen monoxide at said active site. Biological *S*-nitrosoglutathione formation must, therefore, proceed by a route other than a radical–radical reaction.

We conclude that no *S*-nitrosoglutathione is formed from $NO^{\bullet}$ and $GS^{\bullet}$ because the interconversion of $GS^{\bullet}$ to $^{\bullet}GS$ [22,23] is very rapid and because the reaction of $NO^{\bullet}$ with $GS^{\bullet}$ is unexpectedly slow.

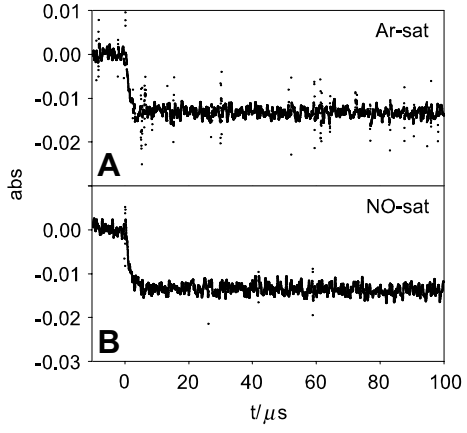

Fig. 1. Laser flash photolysis observed at 330 nm of 1.2×10^{-3} M *S*-nitrosoglutathione in argon (A) and nitrogen monoxide (B) saturated solutions. Both experiments show a bleaching after the laser pulse without relaxation within 10^{-4} s. Even at a nitrogen monoxide concentration of 1.8 mM, the glutathione thiyl radical and nitrogen monoxide radicals do not appear to recombine.

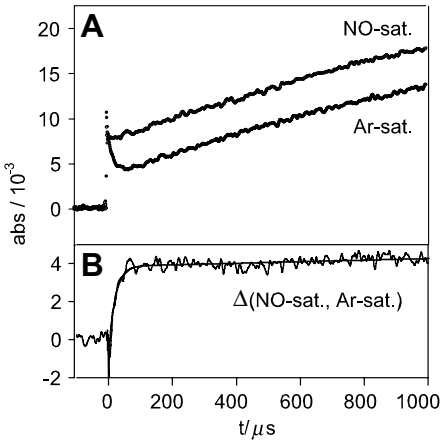

Fig. 2. Laser flash photolysis observed at 330 nm of 2×10^{-3} M oxidised glutathione in argon and nitrogen monoxide saturated solutions, respectively (A). Both experiments show an initial decay and a subsequent build-up. The difference between the traces (B) shows that there is an additional formation process that takes place under nitrogen monoxide only.

Acknowledgments

This work was supported by the ETH Zurich and the Swiss National Science Foundation.

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