Analytical Biochemistry 427 (2012) 36–40

Contents lists available at SciVerse ScienceDirect

Analytical Biochemistry

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

Sulfite species enhance carbon monoxide release from CO-releasing molecules: Implications for the deoxymyoglobin assay of activity

Samantha McLean [a,*], Brian E. Mann [b], Robert K. Poole [a]

[a] Department of Molecular Biology and Biotechnology, University of Sheffield, Western Bank, Sheffield S10 2TN, UK
[b] Department of Chemistry, University of Sheffield, Western Bank, Sheffield S10 2TN, UK

ARTICLE INFO

Article history:
Received 16 March 2012
Received in revised form 10 April 2012
Accepted 24 April 2012
Available online 2 May 2012

Keywords:
CO-releasing molecules
Myoglobin
Hemoglobin
Carbon monoxide
Dithionite

ABSTRACT

Carbon monoxide-releasing molecules (CO-RMs) emulate the beneficial (e.g., anti-inflammatory) effects of CO in biology. CO release from CO-RMs is routinely determined in the presence of reduced deoxy-myoglobin by measuring the formation of carboxy-myoglobin (Mb-CO). Previous studies have highlighted discrepancies between the apparent CO release rates of some CO-RMs established using this assay versus other experimental data where a slower or more complex mechanism of release is suggested. It has been hypothesized that some CO-RMs require a CO acceptor, believed to be reduced myoglobin in Mb-CO assays, in order to facilitate the release of CO. Here, we show, for the first time, that CO is not liberated from the ruthenium (Ru)-based $[Ru(CO)_3Cl_2]_2$ (CORM-2) and $[Ru(CO)_3Cl(glycinate)]$ (CORM-3) at an appreciable rate in the presence of reduced myoglobin alone. Rather, we confirm that it is the reducing agent sodium dithionite that facilitates release of CO from these CO-RMs. Other sulfite compounds, namely sodium sulfite and potassium metabisulfite, also promote the liberation of CO from CORM-3. We describe an alternative oxy-hemoglobin assay that eliminates dithionite and suggest that the efficacy of CO-RMs results from intracellular interactions with anions that facilitate CO delivery to therapeutic targets.

Carbon monoxide is a respiratory inhibitor but is also generated from the breakdown of heme by heme oxygenases. The CO generated has numerous remarkable effects, including anti-inflammatory, signaling, and bactericidal activities [1,2]. However, the use of CO therapeutically is difficult; the gas is potentially toxic and can be delivered only systemically [3]. CO-releasing molecules (CO-RMs)[1] have been developed for more than 10 years in an attempt to emulate the beneficial properties of CO while minimizing toxicity and allowing site-specific delivery. There is now a wide variety of carbonyl compounds with centers including ruthenium, iron, manganese, and boron [4]. Their CO release rates and kinetics are also diverse, with half-lives of seconds to hours. CO-RMs liberate CO in a variety of conditions, in biological buffers [5], by photo dissociation [6], and via enzyme activity [7].

The almost universally used test for CO release is the carboxy-myoglobin (Mb-CO) assay, where CO-RM is added to a solution of reduced deoxy-Mb and the formation of Mb-CO is followed spectrophotometrically [8], exploiting optical changes on CO binding in the α or β regions [9] or the Soret regions [8]. However, recent work has highlighted important discrepancies in the CO release rates of two CO-RMs, $[Ru(CO)_3Cl_2]_2$ (CORM-2) and $[Ru(CO)_3Cl(glycinate)]$ (CORM-3) [5]; CO release assayed by the Mb-CO assay did not correlate with CO release measured directly using a CO electrode. The absence of CO release from CORM-3 has also been demonstrated in the presence of proteins in a study using a gas chromatography thermal conductivity detector [10].

The current study examined the cause of the discrepancy and reveals that the reducing agent sodium dithionite, not myoglobin, is responsible for rapid CO release from Ru-based CO-RMs in the Mb-CO assay. Dithionite also enhances CO release rates from other CO-RMs. We instead propose the use of oxy-hemoglobin as a convenient assay for CO release from CO-RMs and suggest how these findings help to explain the efficacy in vivo of diverse CO-RMs.

Materials and methods

Preparation of CO, CO-RMs, and sodium dithionite solutions

CO-saturated solutions were prepared by bubbling K phosphate buffer (KPi, pH 7.4) with CO for 30 min and were used immediately.

* Corresponding author. Fax: +44 114 2222800.
E-mail address: s.mclean@sheffield.ac.uk (S. McLean).

[1] *Abbreviations used:* CO-RM, CO-releasing molecule; Mb-CO, carboxy-myoglobin; CORM-2, $[Ru(CO)_3Cl_2]_2$; CORM-3, $[Ru(CO)_3Cl(glycinate)]$; KPi, K phosphate buffer; DMSO, dimethyl sulfoxide; CORM-401, $[Mn(CO)_4\{S_2CNMe(CH_2CO_2H)\}]$; PBS, phosphate-buffered saline; Hb, hemoglobin; NAC, *N*-acetylcysteine; NADH, reduced nicotinamide adenine dinucleotide.

0003-2697/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ab.2012.04.026

Such solutions contained 1.15 mM CO measured by the conversion of reduced deoxy-Mb to Mb-CO. CORM-3 was prepared as described previously [11]. CORM-2 (Sigma) was dissolved in dimethyl sulfoxide (DMSO, 10 mM) and used within 15 min. $[Mn(CO)_4\{S_2CNMe(CH_2CO_2H)\}]$ (CORM-401) [12] was dissolved in nitrogen-sparged phosphate-buffered saline (PBS, pH 7.4) to a stock concentration of 1 mM. All CO-RM solutions were kept dark. Sodium dithionite (Sigma, 85%) was prepared as a 100-mM stock solution in 0.1 M KPi (pH 7.4) in an anaerobic cabinet. Additions were performed under anaerobiosis, and cuvettes were sealed before removal from the cabinet. Where excess dithionite was required, grains were added directly to the cuvette.

Deoxymyoglobin–carbonmonoxy-myoglobin assay of CO

CO difference spectra were taken using an Olis RSM1000 dual-beam rapid scanning monochromator (On-Line Instrument Systems). Horse heart Mb (10 μM, Sigma, ⩾90%) in 0.1 M KPi (pH 7.4) was reduced as described. The sample was split into two cuvettes; to one cuvette, 8 μM CO-RM was added and rapidly mixed before recording difference spectra (reduced Mb plus CO-RM minus reduced Mb). ΔA (422–438 nm) measurements gave the concentration of Mb-CO formed over time using the extinction coefficient: Δ_ε (max–min) = 177 mM^{-1} cm^{-1} [13]. Curves were fitted using Microsoft Excel Solver. Where indicated, CO-RM was added to buffer and incubated before adding reduced Mb. For CORM-401 assays, 3 μM CO-RM was used with 13 μM Mb and KPi was substituted by PBS.

Stopped-flow analysis of CO release

Stopped-flow analyses were performed using an Olis RSM1000 spectrophotometer fitted with an Olis USA stopped-flow accessory (On-Line Instrument Systems) employing syringes allowing a 5:1 mixing ratio between protein and ligand solutions. Mb (final concentration 10 μM) was mixed with 8 μM CORM-3 preincubated for 10 min with 0.1 M KPi (pH 7.4) and a molar excess of sodium dithionite. Spectra were taken every millisecond for 2 s. Scans of absolute spectra were subtracted from a scan of 10 μM reduced Mb to produce CO-reduced minus reduced difference spectra. The corresponding concentrations of Mb-CO formed were calculated and plotted versus time.

Oxyhemoglobin–carbonmonoxy-hemoglobin assay of CO

Assays were performed essentially as above. Bovine blood hemoglobin (Hb, Sigma, grade ⩾ 90%) was reduced with dithionite and desalted on a PD-10 column (GE Healthcare), producing oxyferrous Hb and no residual dithionite. Release of CO from CO-RMs was observed by the changes in the Soret region of the spectra due to displacement of oxygen from Hb by CO.

Results

CO is released from CORM-3 in the absence of myoglobin

To assay CO release, we first employed the Mb-CO assay, measuring in the Soret region. Spectra of the CO-bound and reduced deoxy-Mb (Fig. 1A, inset) were plotted in tandem as difference spectra (CO-reduced minus reduced Mb) in split-beam mode (Fig. 1A).

We assayed CO release by the addition of CORM-3 to reduced Mb in the presence of excess dithionite and recorded the concentration of Mb-CO formed as a function of time after the addition of CORM-3 (Fig. 1B, closed circles). CO release from CORM-3 was first order, with a half-life of 2.3 min, in agreement with Clark et al. [11]. Next, we tested whether CO was released from CORM-3 in the absence of Mb. To do this, CORM-3 was incubated in buffer with excess dithionite for 10 min, after which 10 μM Mb was added. Surprisingly, there was an immediate jump in Mb-CO formation on Mb addition, followed by a slower increase over the next hour, ultimately giving the same yield of Mb-CO as when Mb and CORM-3 were added together (Fig. 1B, open circles). Thus, CO is released into solution from CORM-3 during a 10-min incubation of Mb and dithionite and is subsequently captured by adding ferrous myoglobin. To confirm this, we used stopped-flow spectrophotometry to determine the rate of Mb-CO formation over the first 2 s after the addition of Mb (Fig. 1B, inset). Here, Mb-CO formation occurred with the same kinetics as when Mb binds free CO in solution (half-time ~77 ms; data not shown [9]), confirming that CO is released from CORM-3 during incubation in buffer in the absence of Mb.

CO release from CORM-3 is dithionite-dependent

This result appears to disagree with a recent publication [5] in which incubation of CORM-3 in buffer alone allowed the liberation of virtually no CO over approximately 80 min. To investigate this discrepancy, CORM-3 was incubated in buffer in the absence of Mb and dithionite for 10 min, followed by the addition of these reagents to begin the assay. There was no rapid Mb-CO formation after the addition of reduced Mb, suggesting that CO release during the 10-min incubation in buffer alone did not occur (Fig. 1B, closed triangles). In this case, the appearance of Mb-CO occurred monotonically as in Fig. 1B, but the yield of Mb-CO was approximately half that when Mb, CORM-3, and dithionite were added simultaneously.

Further assays were performed anaerobically in the absence of dithionite by prior reduction of the myoglobin followed by dithionite removal. Here, no evidence of CO liberation from CORM-3 was seen over approximately 1 h (data not shown). This demonstrates that dithionite, not Mb, is responsible for facilitating the release of CO from CORM-3. Mb-CO assays were also performed at various dithionite concentrations, confirming that the amount of CO released correlates with the dithionite level in a concentration-dependent manner (see Suppl. Table 1 in Supplementary material).

CO release from CORM-2 is also dependent on the presence of dithionite

To test the generality of this result, we also tested CORM-2, a widely used lipid-soluble, Ru-based CO-RM. When Mb and excess dithionite were added to the assay simultaneously, CO release was first order, with a half-life of 0.5 min, in agreement with Motterlini et al. [14] (Fig. 2A, closed circles). However, when CORM-2 was incubated for 10 min in the presence of dithionite, very rapid Mb-CO formation was immediately apparent on adding Mb, followed by a slower release of CO, qualitatively similar to the results seen with CORM-3 (Fig. 2A, open circles). When CORM-2 was incubated for 10 min in buffer without dithionite and Mb, which were subsequently added together to start the assay, there was no evidence of CO release during the 10-min incubation. Here, as with CORM-3, CO release does not begin until dithionite and Mb are added (Fig. 2A, closed triangles).

CO release from CORM-401 is enhanced by dithionite

Because dithionite is necessary for the fast release of CO from Ru-based CO-RMs, we tested the release of CO from CORM-401, a water-soluble, Mn-based CO-RM, which is reported to release 3.2 molar equivalents of CO per molecule [12]. Due to the greater CO release ratio, Mb-CO assays were conducted with 3 μM CORM-401 in the presence of 13 μM Mb. When all reagents were