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GC-MS determination of nitrous anhydrase activity of bovine and human carbonic anhydrase II and IV

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ABSTRACT

The most widely recognized activity of the large family of the metalloenzyme carbonic anhydrases (CAs) is the diffusion-controlled hydration of CO_2 to HCO_3^- and one proton, and the less rapid dehydration of HCO_3^- to CO_2: $CO_2 + H_2O \leftrightarrows HCO_3^- + H^+$. CAs also catalyze the reaction of water with other electrophiles such as aromatic esters, sulfates and phosphates, thus contributing to lending to CAs esterase, sulfatase and phosphatase activity, respectively. Renal CAII and CAIV are involved in the reabsorption of nitrite, the autoxidation product of the signalling molecule nitric oxide (·NO): $4\ {\cdot}NO + O_2 + 2\ H_2O \rightarrow 4\ ONO^- + 4\ H^+$. Bovine and human CAII and CAIV have been reported to exert nitrite reductase and nitrous anhydride activity: $2\ NO_2^- + 2\ H^+ \leftrightarrows [2\ HONO] \leftrightarrows N_2O_3 + H_2O$. In the presence of L-cysteine, ·NO may be formed. In the literature, these issues are controversial, mainly due to analytical shortcomings, i.e., the inability to detect authentic HONO and N_2O_3. Here, we present a gas chromatography-mass spectrometry (GC-MS) assay to unambiguously detect and quantify the nitrous anhydrase activity of CAs. The assay is based on the hydrolysis of N_2O_3 in $H_2{}^{18}O$ to form $ON^{18}O^-$ and $^{18}ON^{18}O^-$. After pentafluorobenzyl bromide derivatization and electron capture negative-ion chemical ionization of the pentafluorobenzyl nitro derivatives, quantification is performed by selected-ion monitoring of the anions with mass-to-charge (*m/z*) ratios of 46 (ONO^-), *m/z* 48 ($ON^{18}O^-$ and $^{18}ONO^-$), *m/z* 50 ($^{18}ON^{18}O^-$) and *m/z* 47 ($O^{15}NO^-$, internal standard).

Carbonic anhydrases (CAs, EC 4.2.1.1) are a family of metalloenzymes which are catalytically effective only with one metal ion bound within the active site cavity [1]. Zn^{2+} is the metal ion which may be present in all six CA genetic families in mammals including the human α-CA family [1]. In addition to the hydration of CO_2 to bicarbonate and proton and the dehydration of bicarbonate to CO_2 (reaction 1: i.e., the carbonic anhydrase activity of CAs) CAs catalyze the reaction of water with several other electrophilic molecules including aromatic (Ar) esters (RCOOAr), phosphates ($ArOPO_3H_2$) and sulfates ($ROSO_3Ar$) (reviewed in Ref. [1]), thus contributing to CA esterase, phosphatase and sulfatase activity, respectively.

$$O{=}C{=}O + H_2O \leftrightarrows O{=}C(\text{-}OH){-}O^- + H^+ \quad (1)$$

Bovine CAII has previously been proposed to exhibit inorganic nitrite reductase activity, i.e., to reduce nitrite ($O{=}N{-}O^-$; N oxidation state +III) to form nitric oxide (·NO; N oxidation state +II), and the CA inhibitor dorzolamide to enhance the nitrite reductase activity of CAII [2,3]. By means of an NO-sensitive electrode we demonstrated that bovine erythrocytic CAII, recombinant human erythrocytic CAII and recombinant human erythrocytic CAIV do not induce release of ·NO from nitrite or $^{15}{\cdot}NO$ from ^{15}N-labelled nitrite ([^{15}N]nitrite) at neutral pH, either in the absence or in the presence of various CA inhibitors including dorzolamide [4,5]. By means of an NO-sensitive electrode, Andring et al. [6] confirmed our previous results for bovine CAII [4,5]. These observations suggest that bovine CAII, human CAII and human CAIV do not exhibit nitrite reductase activity. This conclusion is to be expected because the zinc cation of CA has only a single oxidation state (Zn^{2+}). We suggest that a possible explanation for the discrepancy in the literature could be the use by Aamand et al. [2] of a pharmaceutical formulation of dorzolamide that contained additional unspecified redox-active ingredients. This explanation is realistic because we found by means of an NO-sensitive electrode that CAII and CAIV can mediate conversion of nitrite to ·NO in the presence of L-cysteine which

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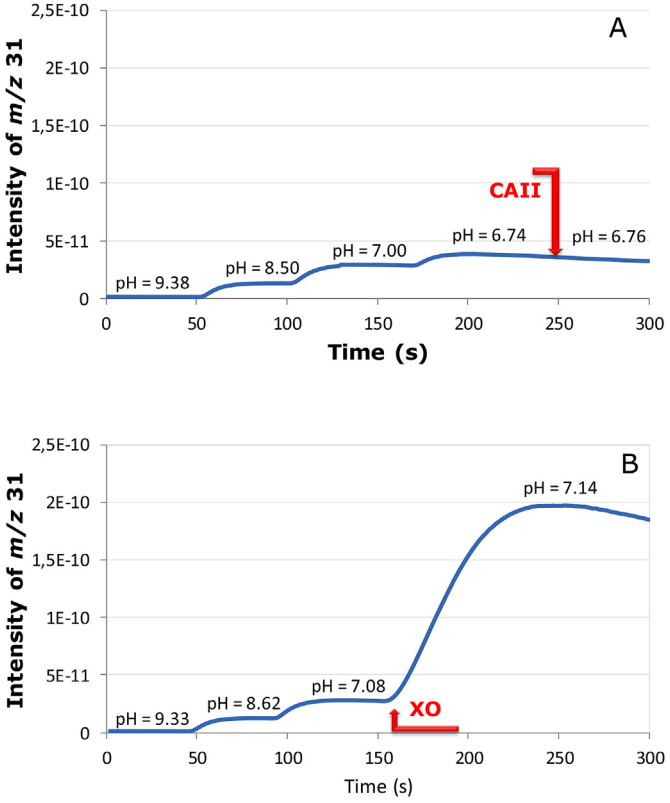

Fig. 1. Representative online MIMS chromatograms from the analysis of ^{15}NO (m/z 31) in aerobic (60 or 75 µM O_2) solutions of 10 mM $Na^{15}NO_2$ in aqueous 25 mM $NaHCO_3$ before and after addition of (A) bovine CAII (concentration, 6 µg/mL) or (B) xanthine (100 µM) and xanthine oxidase (concentration, 132 µg/mL) as indicated by the arrows. MIMS analyses were performed as reported elsewhere [7].

contains a readily oxidizable sulfhydryl (SH) group [4].

Membrane inlet mass spectrometry (MIMS) is an alternative technique to detect gases such as CO_2 and ·NO, and possibly of HONO, ·NO_2 and N_2O_3 (nitrous anhydride). Upon introduction of gaseous molecules into the mass spectrometer, they are converted by electron ionization to cations which can be identified according to their mass-to-charge (m/z) ratios. MIMS is most useful to assay CA activity by online monitoring of CO_2 (as $[CO_2]^{\cdot +}$, m/z of 44) and ^{18}O-labelled CO_2 ([as $^{18}OCO]^{\cdot +}$, m/z of 46) when the reaction is performed in $H_2{}^{18}O$ [7]. Based on the lack of MIMS signals at m/z 30 (NO^+), m/z 46 ($NO_2{}^+$) and m/z 76 ($[N_2O_3]^+$) for ·NO, ·NO_2 and N_2O_3, respectively, Andring et al. concluded that purified bovine CAII does not exhibit nitrite reductase or nitrous anhydrase activity in aqueous buffer at pH 5.9 in the absence or in the presence of dorzolamide [6]. By MIMS we also found no formation of ^{15}·NO from [^{15}N]nitrite in aqueous buffer by CAII, whereas xanthine oxidase (XO) was found to reduce [^{15}N]nitrite to ^{15}·NO, albeit to a very low extent (Fig. 1).

It is worth mentioning that MIMS seems to be not sensitive enough to measure ·NO, HONO, ·NO_2 and N_2O_3 in aqueous buffered solutions of nitrite at neutral pH [7–11]. The limitations of the MIMS technique to detecting N_2O_3 have been discussed by Tu et al. [10] and Mikulski et al. [11]. To the best of our knowledge, the detection of authentic HONO and N_2O_3 in aqueous buffered solutions of nitrite by MIMS or other mass spectrometry techniques has not been reported thus far (see Refs. [9–12]). Given these serious analytical limitations of MIMS, the very weak and almost unchanged signals for m/z 46 ($NO_2{}^+$) and m/z 76 ($[N_2O_3]^{\cdot +}$) in the study by Andring et al. [6] do not necessarily exclude bovine CAII-mediated formation of N_2O_3 from nitrite in buffered solutions and do not definitely preclude the possibility that bovine CAII possesses nitrous anhydrase activity. More sensitive analytical methods are required to address these issues. Direct HONO and N_2O_3 measurements are currently beyond current MS capabilities. However, alternative but indirect approaches, based on the measurement of analytically accessible stable and specific reaction products of HONO and N_2O_3, are more promising.

HONO and N_2O_3 are strong nitrosating species and induce *N*–, *S*– and *C*-nitrosation of amines (RNH_2, reaction 2), thiols such as free L-cysteine or L-cysteine moieties in proteins and peptides (e.g., glutathione (GSH), γ-L-glutamyl-L-cysteinyl-glycine, reaction 3), and activated aromatic compounds such as free L-tyrosine (L-TyrH, reaction 4) and L-tyrosine residues in proteins, respectively. Thus, formation for *S*-nitrosoglutathione (GSNO) in aqueous buffered solutions of nitrite, GSH and CA at neutral pH would strongly indicate formation of HONO and/or N_2O_3, and its formation rate would correspond to CA nitrous anhydrase activity. Indeed, by means of a highly specific and sensitive liquid chromatographic-tandem mass spectrometric (LC-MS/MS) method for GSNO [13], we observed CAII-induced formation of GSNO in the presence of GSH [5]. In the presence of L-cysteine (L-CysSH) instead of GSH, formation of *S*-nitroso-L-cysteine (L-CysSNO, reaction 5) would be expected. However, L-CysSNO is very labile and rapidly decomposes to ·NO [14]. Indeed, by means of an ·NO-sensitive electrode, we observed CAII- and CAIV-mediated conversion of nitrite to ·NO in the presence of L-cysteine [4]. Thus, formation of ·NO in aqueous buffered solutions of nitrite and CA would be indicative of HONO and/or N_2O_3 formation due to CA nitrous anhydrase activity.

$$HONO + RNH_2 \rightarrow RN(NO)H + H_2O \quad (2)$$

$$HONO + GSH \rightarrow GSNO + H_2O \quad (3)$$

$$HONO + L\text{-}TyrH \rightarrow L\text{-}TyrNO + H_2O \quad (4)$$

$$HONO + L\text{-}CysSH \rightarrow L\text{-}CysSNO + H_2O \quad (5)$$

N2O3 is assumed to occur in four isomeric structures [15]. The most realistic structure of N_2O_3 formed from dehydration of nitrous acid in aqueous solutions is symmetric O═N–O–N═O [16]. In contrast, N_2O_3 formed from the combination of the radicals ·NO and ·NO_2 in gaseous phase is more likely to have the structure O═N–N(═O)O [16]. O═N–O–N═O and O═N–N(═O)O may coexist in aqueous solution as an equilibrium mixture [11]. Oxidation of ·NO in water free of transition metal cations such as Fe^{3+} and Cu^{2+} yields exclusively nitrite (see reaction 6) [8,17,18] and is likely to occur in two steps: first, oxidation of ·NO by molecular oxygen to generate O═N–O–N═O (reaction 6a) and then its subsequent hydrolysis to form nitrite (reaction 6b). By contrast, O═N–N(═O)O is assumed to dissociate to ·NO and ·NO_2 [1]. ·NO_2 would disproportionate in aerobic aqueous solutions to generate nitrite and nitrate. Yet, nitrate is not an oxidation product of ·NO in aqueous solution in the absence of redox-active metal cations and heme groups [8,17,18]. Therefore, O═N–O–N═O seems to better fit experimental observations and the chemistry of ·NO in aqueous aerated solutions [8,17,18].

$$4\ {\cdot}N{=}O + O_2 + 2\ H_2O \rightarrow 4\ O{=}N{-}O^- + 4\ H^+ \quad (6)$$

$$4\ {\cdot}N{=}O + O_2 \rightarrow 2\ O{=}N{-}O{-}N{=}O \quad (6a)$$

$$2\ O{=}N{-}O{-}N{=}O + 2\ H_2O \rightarrow 4\ O{=}N{-}O^- + 4\ H^+ \quad (6b)$$

At neutral pH, nitrous acid ($pK_a \approx 3.4$) is almost entirely dissociated to nitrite (reaction 7), and only a very small fraction of nitrous acid can dehydrate to form nitrous anhydride which is likely to hydrolyse back to nitrous acid (reaction 8) and to react with nucleophiles such as the SH group of GSH to form GSNO (reaction 9), analogous to nitrous acid (reaction 3). Thus, CA-induced conversion of nitrite to GSNO would not be specific for nitrous acid or nitrous anhydride formation. On the other hand, measurement of nitrite in the absence of L-cysteine would not allow assay nitrous anhydrase activity of CA.

$$O{=}N{-}OH \leftrightarrows O{=}N{-}O^- + H^+ \quad (7)$$