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Comment on the article *Structure and mechanism of copper–carbonic anhydrase II: a nitrite reductase*

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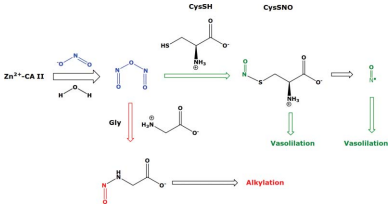
Carbonic anhydrase (CA) is one of the oldest, most efficient and best investigated ubiquitous Zn^{2+}-containing enzymes. CA catalyzes a very simple but vital reaction, *i.e.* the hydration of carbon dioxide, in mammals, plants and bacteria (Meldrum & Roughton, 1933). Rather surprisingly, over recent decades many additional physiological and pathological roles of CA have been discovered. A newly discovered CA activity is the bioactivation of inorganic nitrite (O═N—O^-) to nitric oxide (NO), a signaling multiple-functional gaseous molecule in living organisms. Central to scientific research on CA has been its catalytic site that preferentially binds Zn^{2+}, which is redox-inactive, and Cu^{2+}, which is redox-active (Lindskog & Nyman, 1964; Coleman, 1965). This topic is still of great scientific interest (Kim *et al.*, 2020). In addition, and in contrast to Zn^{2+}, Cu^{2+} binds to two different centers of CA which are differently affected by glutathione (GSH), the most abundant endogenous intra-cellular antioxidant with high specificity to Zn^{2+}, Cu^{2+} and other divalent ions including Hg^{2+} (Tabbì *et al.*, 2019). It can be expected that Cu^{2+}-carrying CA is likely to exert not only the classical carbonic anhydrase activity, but may also be involved in redox-dependent reactions and mechanisms. For example, Cu^{2+}-containing CA could oxidize NO to nitrite and higher nitrogen oxides (NO_x), as performed by the Cu^{2+}-rich ceruloplasmin, or it could reduce nitrite to NO via intermediate Cu^+-formation by GSH or ascorbic acid (Tabbì *et al.*, 2019). Such a reaction is practically impossible for regular Zn^{2+}-containing CA.

Recently, Andring and associates reported the crystal structure of copper (II)-bound human carbonic anhydrase II (Cu^{2+}-hCAII) in complex with inorganic nitrite (O═N—O^-) at 1.2 Å resolution with two Cu^{2+} centers, analogous to bacterial nitrite reductases, and suggested that Cu^{2+}-hCAII can function as a nitrite reductase, yet without providing experimental evidence (Andring *et al.*, 2020). In the *scientific commentary* on this article, Liljas stated that 'Andring *et al.* (2020) have been able to unravel the mystery' (Liljas, 2020), probably referring to the controversy that Aamand *et al.* (2009) found Zn^{2+}-CAII to reduce nitrite to NO, whereas Andring *et al.* (2018) failed to detect Zn^{2+}-CAII-mediated reduction of nitrite to NO.

Our studies using bovine and human Zn^{2+}-CAII demonstrated formation of *S*-nitrosoglutathione (GSNO) from nitrite and GSH suggesting nitrous anhydrase activity of Zn^{2+}-CAII, which was not inhibitable by the CA-inhibitors acetazolamide or dorzolamide (Hanff *et al.*, 2016; Zinke *et al.*, 2016). We observed formation of NO only in the presence of L-cysteine (CysSH), most likely due to the intermediate formation of *S*-nitrosocysteine (CysSNO), which can readily and abundantly decompose to NO in the presence of Cu^+ (Tsikas *et al.*, 2002).

Cu^{2+} ions were found to bind to Zn^{2+}-CAII isolated from human erythrocytes at a site other than the active site and inhibited the exchange of water from the enzyme without affecting the equilibrium rate of hydration of CO_2 (Tu *et al.*, 1981). This observation may suggest that classical CA inhibitors such as acetazolamide may inhibit the carbonic anhydrase activity of CA by tightly binding to the CAII-bound Zn^{2+}, through the sulfone amide group, but not to the second Cu^{2+}-binding site. This could be an explanation for our observation that neither acetazolamide nor dorzolamide inhibited the nitrous anhydrase activity of bovine and human CAII (Hanff *et al.*, 2016; Zinke *et al.*, 2016).

Andring *et al.* (2020) stated that 'recent reports have shown that CAII can also reduce nitrite (NO_2^-) to nitric oxide (NO)... (Andring *et al.*, 2018; Aamand *et al.*, 2009; Hanff *et*



al., 2018)', that 'However, when dialyzed with ethylenediaminetetraacetic acid (EDTA), the enzyme retained its carbonic anhydrase activity yet lost its nitrite reductase activity (Hanff *et al.*, 2018)', and that 'Furthermore, if this bovine CAII was dialyzed against EDTA, the nitrite reductase activity was ablated indicating that a metal cofactor within the bovine blood was needed for the CAII-dependent nitrite reductase activity (Andring *et al.*, 2018; Hanff *et al.*, 2018).'. We wish to point out this mistake in the paper by Andring *et al.* (2020). In the paper referred to above (*i.e.*, Hanff *et al.*, 2018), we did not report that CAII is a nitrite reductase, but we explicitly stated that we measured nitrous anhydrase activity of bovine and human CAII and CAIV, and did not use EDTA (*i.e.* Hanff *et al.*, 2018).

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