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Bioenergetic relevance of hydrogen sulfide and the interplay between gasotransmitters at human cystathionine β-synthase

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ABSTRACT

Merely considered as a toxic gas in the past, hydrogen sulfide (H_2S) is currently viewed as the third 'gasotransmitter' in addition to nitric oxide (NO) and carbon monoxide (CO), playing a key signalling role in human (patho)physiology. H_2S can either act as a substrate or, similarly to CO and NO, an inhibitor of mitochondrial respiration, in the latter case by targeting cytochrome *c* oxidase (CcOX). The impact of H_2S on mitochondrial energy metabolism crucially depends on the bioavailability of this gaseous molecule and its interplay with the other two gasotransmitters. The H_2S-producing human enzyme cystathionine β-synthase (CBS), sustaining cellular bioenergetics in colorectal cancer cells, plays a role in the interplay between gasotransmitters. The enzyme was indeed recently shown to be negatively modulated by physiological concentrations of CO and NO, particularly in the presence of its allosteric activator S-adenosyl-L-methionine (AdoMet). These newly discovered regulatory mechanisms are herein reviewed.
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1. Hydrogen sulfide synthesis and breakdown in human physiology

Hydrogen sulfide (H_2S) has emerged in the past decades as the third recognized 'gasotransmitter' in human physiology. Much like its 'predecessors' nitric oxide (NO) and carbon monoxide (CO), H_2S was historically considered a poisonous gas that was subsequently discovered to be endogenously synthesized to fulfil a myriad of signalling and regulatory functions [1–4]. This colourless gas with a strong characteristic odor (the smell of rotten eggs) is slightly polar, which allows it to freely diffuse across biological milieu, including membranes. In aqueous solution, H_2S equilibrates with hydrosulfide (HS^-) and sulfide (S^{2-}), according to the pK_{a1} ~7.0 (H_2S/HS^-) and pK_{a2} ~19 (HS^-/S^{2-}) measured at 25 °C. At a 'physiological' pH of 7.4, HS^- is the predominant species (70–80%) and H_2S occurs at 20–30%, whereas S^{2-} is supposedly present in negligible amounts [3,5,6]. Unless otherwise stated, the terms 'H_2S' and 'sulfide' are herein employed interchangeably to collectively indicate the H_2S/HS^- pair.

H_2S has a dose-dependent effect on multiple physiological processes, acting as a signalling molecule (mostly resulting in persulfidation of cysteine residues in proteins [4,5,7]) and a bioenergetic 'fuel' at low concentrations, while displaying deleterious effects at high concentrations. Although the *in vivo* free H_2S levels in mammalian physiology are difficult to estimate and remain a matter of debate, it is presently accepted that high nanomolar to low micromolar concentrations may occur under non-pathological conditions [8]. Disturbances in H_2S metabolism either impairing or enhancing H_2S production and/or breakdown are currently and growingly associated with many pathological conditions, ranging from cardiovascular [9] and neurodegenerative diseases [10] to cancer [11].

H_2S is generated in humans via two major routes: ubiquitous endogenous enzymes (described below) and the gut microbiota, particularly sulfate-reducing bacteria. The three major human enzymes recognized as endogenous H_2S sources are the methionine cycle transsulfuration pathway enzymes cystathionine β-synthase (CBS) and cystathionine γ-lyase (CGL), and the 3-mercaptopyruvate sulfurtransferase (MST) (Fig. 1A) [1,12]. Whereas CBS and CGL are both pyridoxal 5′-phosphate (PLP)-dependent enzymes, MST has a single redox-active cysteine

Abbreviations: H_2S, hydrogen sulfide; NO, nitric oxide; CO, carbon monoxide; CcOX, cytochrome *c* oxidase; CBS, cystathionine β-synthase; AdoMet, S-adenosyl-L-methionine; CGL, cystathionine γ-lyase; PLP, pyridoxal 5′-phosphate; MST, 3-mercaptopyruvate sulfurtransferase; CAT, cysteine aminotransferase; 3-MP, 3-mercaptopyruvate; SOU, sulfide oxidizing unit; SQR, sulfide:quinone oxidoreductase; GSH, reduced glutathione, GSSH, glutathione persulfide; ETHE1, persulfide dioxygenase; Rhod, rhodanese; SOx, sulfite oxidase; FCCP, *p*-trifluoromethoxyphenylhydrazone, MSR, methionine synthase reductase; AOAA, aminooxyacetic acid; AdoHcy, S-adenosyl-L-homocysteine.

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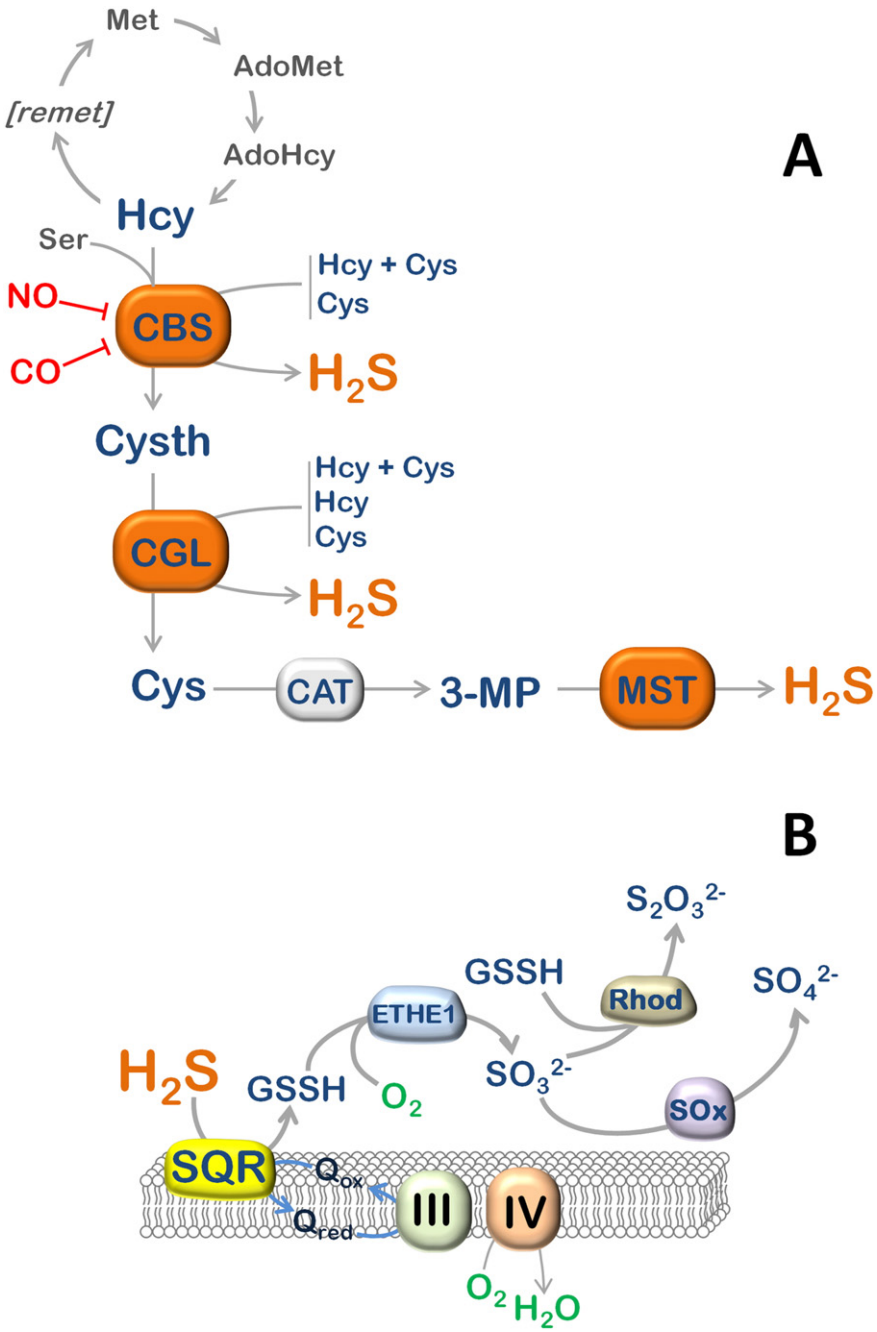

Fig. 1. H_2S metabolism in human physiology. *Panel A*, H_2S biosynthetic pathway. Homocysteine (Hcy) is generated through the methionine cycle, involving the sequential conversion of methionine (Met) into S-adenosyl-L-methionine (AdoMet), followed by AdoMet demethylation into S-adenosyl-L-homocysteine (AdoHcy) and production of Hcy by hydrolysis. Cystathionine β-synthase (CBS) catalyzes the condensation of serine (Ser) and Hcy into cystathionine (Cysth) and water. Alternative reactions catalyzed by CBS, using cysteine (Cys) alone or Cys and Hcy, result in H_2S production. The next step in the transsulfuration pathways is catalyzed by cystathionine γ-lyase (CGL), which converts Cysth into Cys. Alternative reactions catalyzed by CGL, using Hcy and/or Cys as substrates, yield H_2S. Cys is converted by cysteine aminotransferase (CAT) into mercaptopyruvate (3-MP), which is used as a substrate by mercaptopyruvate sulfurtransferase (MST) for H_2S production. *Panel B*, sulfide oxidizing unit: H_2S is oxidized by sulfide:quinone oxidoreductase (SQR), with reduced glutathione (GSH) as the preferred sulfur acceptor, yielding glutathione persulfide (GSSH) and reduced quinone (Q). GSSH is catabolized by persulfide dioxygenase (ETHE1) into sulfite, using O_2 as co-substrate. GSSH and sulfite are converted by rhodanese (Rhod) into thiosulfate. Finally, sulfite is oxidized to sulfate by sulfite oxidase (SOx). Reduced quinone (Q_{red}) supplies electron equivalents via Complex III to accomplish Complex IV-mediated oxygen respiration.

residue in the active site. As mentioned above, both CBS and CGL participate in the transsulfuration pathway of methionine metabolism. Therefore, their physiological relevance is both set on homocysteine homeostasis and on H_2S generation [13]. Indeed, both enzymes produce H_2S via reactions that are alternative to their historically-considered canonical activities. The CBS canonical activity concerns the condensation of serine and homocysteine into cystathionine and water, but the enzyme can also use cysteine alone or together with homocysteine to generate H_2S and different co-products [13] (Fig. 1A). Similarly, CGL, whose canonical reaction is the conversion of cystathionine into cysteine with the production of α-ketobutyrate and ammonia, can generate H_2S from cysteine and/or homocysteine [13] (Fig. 1A). This seemingly catalytic promiscuity may be viewed as a robust system to ensure H_2S generation from different substrate combinations and under several physiological conditions. MST functions together with another enzyme, cysteine aminotransferase (CAT), which converts cysteine and α-ketoglutarate into glutamate and 3-mercaptopyruvate (3-MP), the latter used as a substrate for the MST-catalyzed H_2S production [1]. An additional pathway

has been recently demonstrated involving MST, where 3-MP alternatively derives from D-cysteine and a D-amino acid oxidase [14]. In terms of cellular localization, the classical view is that CBS and CGL are cytosolic enzymes, whereas MST is located in the mitochondria. However, there are numerous exceptions where both CBS and CGL can be found in mitochondria and MST in the cytosol, which preclude taking a firm position in this regard. Examples of CBS localization in the mitochondria and its impact on cellular bioenergetics and mitochondrial function are detailed below.

As any other potentially toxic gaseous molecule endogenously synthesized with signalling purposes, H_2S levels must be kept under strict control. Indeed, as reviewed in [1,15], human cells are equipped with an H_2S breakdown enzymatic system associated to the mitochondria (Fig. 1B), initially identified in the lugworm *Arenicola marina* [16], and more recently denoted as 'sulfide oxidizing unit' (SOU) [17]. The enzymatic unit comprises a sulfide:quinone oxidreductase (SQR), a persulfide dioxygenase (also known as ETHE1 or sulfur dioxygenase), a thiosulfate sulfurtransferase (rhodanese, Rhod) and a sulfite oxidase (SOx), that overall catalyze the breakdown of H_2S to thiosulfate and sulfate, the main sulfide catabolites (Fig. 1B). A more detailed description of the mitochondrial SOU is given in Section 2.3.

2. Hydrogen sulfide and mitochondrial respiration

As a common feature, the three gasotransmitters NO, CO and H_2S can all inhibit mitochondrial cytochrome *c* oxidase (CcOX), causing a decline in the electron flux of the respiratory chain and thus in ATP synthesis (reviewed in [18], Fig. 2). NO can also inhibit Complex I *via* S-nitrosation [19–22], the inhibition being dependent on a reversible conformational change of the enzyme [23]. More recent studies, however, have shown that, unlike the other two gasotransmitters, at low concentrations (nanomolar to low micromolar) H_2S can also stimulate mitochondrial respiration by acting as a mitochondrial electron donor [17,24–30] (Fig. 2). This makes H_2S a very interesting molecule from a bioenergetic viewpoint, able to induce opposite effects on mitochondrial respiration depending on its bioavailability (reviewed in [8,15,31,32]).

2.1. H_2S as an inhibitor of mitochondrial respiration

Cytochrome *c* oxidase (CcOX), considered as the main target of gasotransmitter-mediated inhibition of mitochondrial respiration, is inhibited by the three gasotransmitters with different kinetics [18]. CO acts a simple competitive inhibitor, lowering the enzyme K_M for O_2 by binding at the O_2-reactive, fully-reduced heme a_3-Cu_B active site ([33], Fig. 3). CcOX inhibition by NO is more complex (reviewed in [34–39]). It can proceed through two alternative reaction pathways [40–45], one competitive and the other uncompetitive towards O_2, i.e., leading to a decrease of both V_{max} and K_M for O_2 yet keeping their ratio unchanged [46,47]. The O_2-competitive pathway, prevailing at higher electron flux and lower O_2 levels, leads to an adduct of CcOX with NO bound to ferrous heme a_3, whereas the O_2-uncompetitive one, favoured at lower electron flux and higher O_2 levels, results into the formation of an inhibited state of the enzyme with nitrite bound to ferric heme a_3 ([18,44,46,48], Fig. 3).

Differently from CO and NO, H_2S was reported to be a non-competitive inhibitor of isolated mitochondrial CcOX [49], i.e., affecting the enzyme V_{max}, but not the K_M for O_2. The inhibition is reversible, potent ($K_i = 0.2$ μM at pH 7.4) and independent of oxygen concentration [49]. Interestingly, sulfide inhibition of CcOX is pH dependent, the K_i dropping from 2.6 μM to 0.07 μM as the pH decreases from 8.05 to 6.28 [50]. Consistently, CcOX inhibition by sulfide is more effective under acidosis conditions (see [8,31] and the references therein). Although the effect of pH has not been investigated in mechanistic details, the enhanced inhibition is likely related to the fact that H_2S, with its pK_a of ~7.0, prevails over HS^- in acidic conditions. The active site of CcOX,

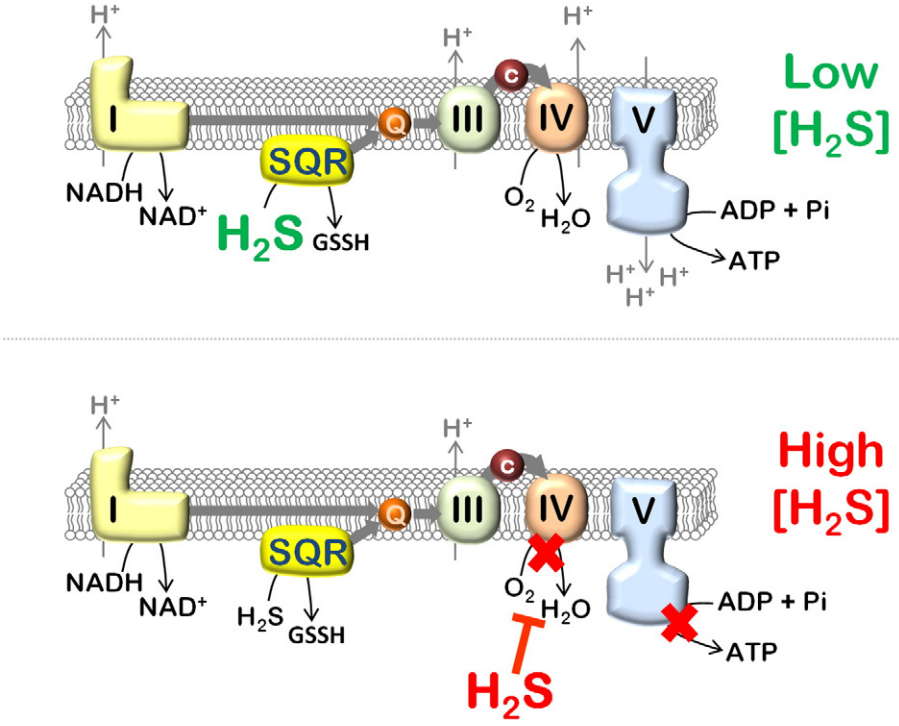

Fig. 2. H_2S and mitochondrial respiration. Dual effect of H_2S on mitochondrial respiration depending on its bioavailability. At low concentrations, H_2S supplies the mitochondrial electron transfer chain, being oxidized by sulfide:quinone oxidoreductase (SQR) with concomitant reduction of coenzyme Q. Reduced quinone (also derived from NADH oxidation by Complex I) is then oxidized by Complex III to generate reduced cytochrome *c*, finally used by Complex IV as the electron source for O_2 reduction to water. Proton translocation by Complexes I, III and IV contribute to Complex V-mediated ATP production. At high H_2S concentrations, cytochrome *c* oxidase (Complex IV) is directly inhibited by H_2S, blocking respiration and concomitant ATP synthesis.

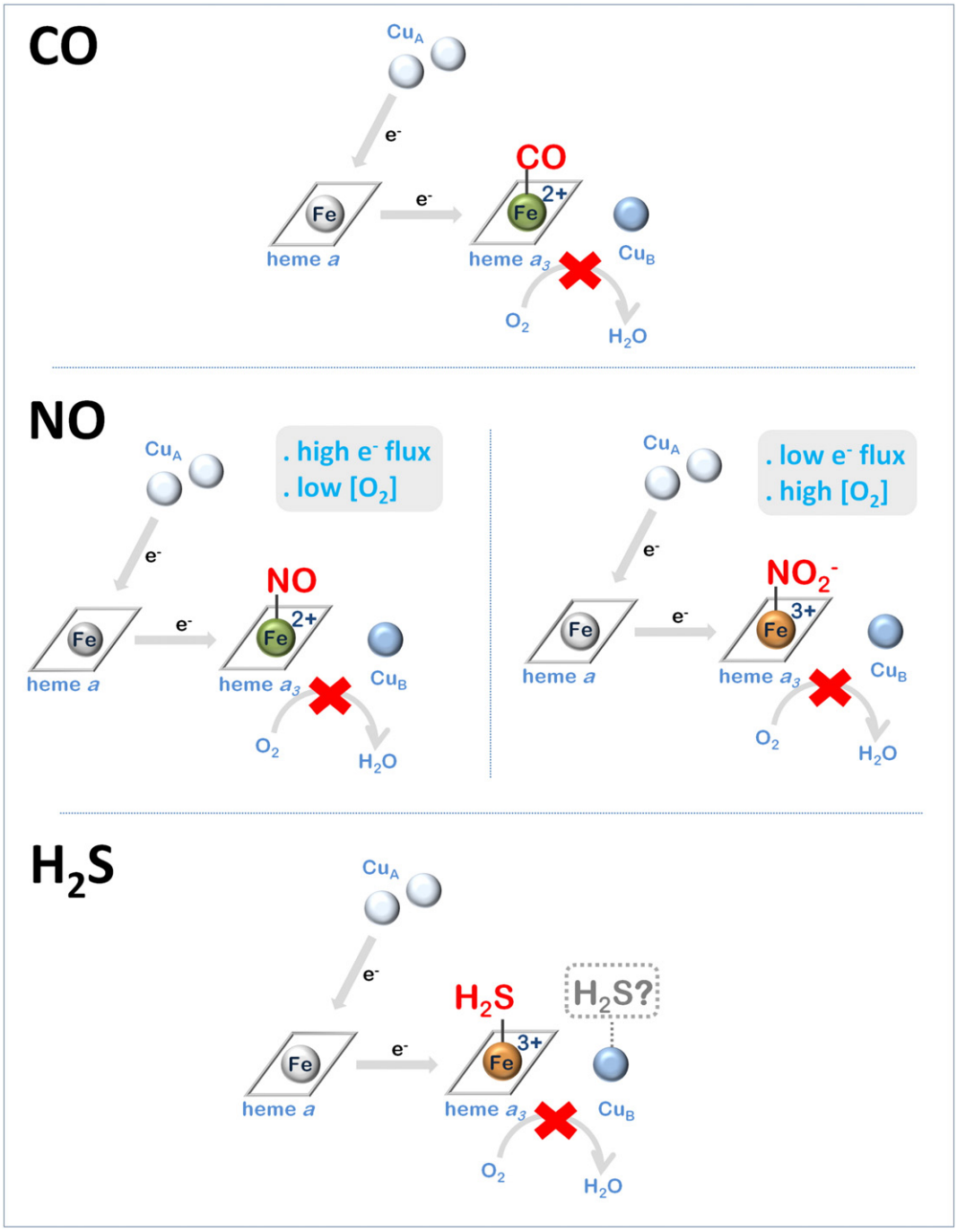

Fig. 3. Inhibition of cytochrome oxidase by the three gasotransmitters CO, NO and H_2S. Redox active metal centres of cytochrome oxidase (CcOX): the binuclear Cu_A centre is the electron entry point, heme *a* mediates electron transfer to the O_2-reducing binuclear active site, composed by heme a_3 and Cu_B. CO inhibits CcOX by tight reversible binding to ferrous heme a_3. The CcOX inhibition pathway by NO varies depending on the electron flux and O_2 level. At high electron flux and low [O_2], NO binds to ferrous heme a_3. At low electron flux and high [O_2], the inhibited adduct has nitrite bound to ferric heme a_3. Sulfide-inhibited CcOX has H_2S bound to ferric heme a_3 and possibly another H_2S molecule bound to cuprous Cu_B.

being located in an apolar environment, is indeed expected to preferentially bind electroneutral species, such as H_2S, or proton-neutralized anionic species ($HS^- + H^+$) [51], both favoured at lower pH. Sulfide inhibition of isolated mitochondrial CcOX is not only effective, but also relatively fast, proceeding with an initial rate constant of $2.2 \times 10^4\ M^{-1}\ s^{-1}$, as measured at pH 7.4 with the enzyme in turnover with O_2 in the presence of ascorbate and cytochrome *c* [52]. Unlike NO and CO [53], H_2S does not bind ferrous heme a_3. The inhibited enzyme displays sulfide bound to ferric heme a_3 (Fig. 3), preventing the reaction with O_2 and thus leading to electron accumulation at the Cu_A and heme *a* sites [54,55]. Based on electron paramagnetic resonance (EPR) spectroscopy [56], Cu_B in sulfide-inhibited CcOX was suggested to be in the cuprous state, possibly bound to a second H_2S molecule (Fig. 3), with a substantial increase of the reduction potential. The mechanism of inhibition is only partly understood. H_2S was suggested to bind the enzyme in turnover at cupric or cuprous Cu_B to be transferred intramolecularly to ferric heme a_3, leading to enzyme inhibition [52].

2.2. H_2S oxidation by mitochondrial cytochrome c oxidase

Somewhat similarly to the other two gasotransmitters, H_2S is not only an inhibitor, but it can also serve as a reducing substrate for CcOX

[50,52,57]. Besides being able to slowly oxidize CO to CO_2 [58,59] and much more quickly NO to nitrite [42,60,61], CcOX can also oxidize sulfide probably to a persulfide species [50,52,57]. Sulfide directly reacts with the oxidized heme a_3-Cu_B active site in the so-called 'pulsed' state of the enzyme [52]. This state is obtained by subjecting to a reduction/oxidation cycle the 'resting' enzyme, much less reactive with external ligands. This 'pulsing' procedure has been discovered in 1977 by Antonini and co-workers [62]. It remains to be demonstrated whether the enhanced reactivity of 'pulsed' CcOX towards H_2S arises from Cl^- dissociation from the active site, as previously shown for NO [63]. With pulsed CcOX, in the absence of reducing substrates, the heme a_3-Cu_B site is directly reduced by low sulfide levels and the produced ferrous heme iron under aerobic conditions is promptly converted into a ferryl state, optically resembling the 'P' catalytic intermediate [52]. This species eventually decays slowly (tens of seconds) into the 'F' ferryl form of the enzyme by reacting with residual sulfide or by auto-reduction [52]. The physiological relevance of this CcOX-mediated oxidative degradation of H_2S remains to be determined, because mitochondrial SQR seems to provide a very effective H_2S dissimilatory pathway, probably out-competing the H_2S-metabolizing activity of CcOX. Finally, it is worth mentioning that H_2S can also directly reduce cytochrome *c* [50]. The reaction, however, is probably too slow and with too low affinity to have a physiological relevance, although to our knowledge it has not yet been investigated with cardiolipin-bound cytochrome *c*, much more reactive towards external ligands [64].

2.3. H_2S as an effective substrate of the mitochondrial respiratory chain

Despite the low K_i value measured with isolated CcOX ($K_i = 0.2$ μM at pH 7.4 [49]), inhibition of respiration in isolated mitochondria or intact cells requires much higher H_2S concentrations (up to tens of micromolar, see for instance [65,66]). This finding is fully consistent with the observation that H_2S at relatively low concentrations (nanomolar to low micromolar) also acts as an effective substrate of the mitochondrial respiratory chain. Evidence for mitochondrial sulfide oxidation associated with oxygen consumption and ATP synthesis was first obtained by investigating the invertebrate *Solemya reidi* [24]. This is the first study in which the dual effect of H_2S on mitochondrial respiration, inhibitory at higher concentration and stimulatory at lower levels, was demonstrated. These initial observations were expanded in more recent studies conducted on isolated mitochondria as well as on permeabilized or intact cells from higher organisms, including human cell lines [17,25–30]. Quantitative information on sulfide oxidation and related bioenergetic effects was often obtained in these studies by supplying sulfide at selected infusion rates rather than as a single bolus, an approach reviewed in [32]. Due to the opposite effects of sulfide on mitochondrial respiration, stimulation of oxygen consumption and mitochondrial energization is best appreciated at low sulfide concentrations, non-inhibitory towards CcOX.

Electrons derived from sulfide oxidation are accepted by coenzyme Q (Fig. 2). Sulfide-stimulated oxygen consumption is indeed blocked by inhibitors of the bc_1 complex (antimycin) or of CcOX (cyanide), being unaffected by rotenone inhibition of Complex I [26,65,67,68], except in colonocytes where Complex I has been suggested to work in a reverse mode [17,26] (see Section 2.4). Based on this and other evidence [16], the existence of a mitochondrial sulfide oxidizing unit (SOU) was envisaged [17]. In such a unit the primary sulfide oxidizing enzyme is the membrane-associated sulfide:quinone oxidoreductase (SQR), having a cysteine disulphide in its active site and harboring a non-covalent flavin adenine dinucleotide moiety from where sulfide-derived electrons are transferred to coenzyme Q [69] (Fig. 1B). H_2S oxidation by human SQR involves the transfer of a sulfur atom to an acceptor which has been a topic of discussion in the literature [70,71]: it was proposed to be i) reduced glutathione (GSH) yielding glutathione persulfide (GSSH), ii) sulphite (SO_3^{2-}) yielding thiosulfate ($S_2O_3^{2-}$) or iii) an unknown acceptor. Recently, it has been proposed that at physiological GSH and sulfite concentrations, GSH is the preferred sulfur acceptor for SQR-mediated H_2S oxidation [71]. The next step in the sulfide oxidation pathway is catalyzed by the persulfide dioxygenase ETHE1 (Fig. 1B), a mononuclear non-heme iron enzyme structurally belonging to the metallo-β-lactamase family, which uses oxygen as a co-substrate for generation of sulfite from GSSH [71–73]. GSSH and sulfite are further converted into thiosulfate and GSH by rhodanese (Rhod, Fig. 1B), having a single redox-active cysteine in its active site [71,74]. Rhodanese catalyzes a myriad of reactions involving numerous substrates, but the transfer of a sulfur atom from GSSH to sulfite to generate thiosulfate appears to be the most catalytically favourable reaction at physiologically relevant substrate concentrations [71]. The last step in the sulfide oxidation pathway is catalyzed by sulfite oxidase (SOx, Fig. 1B), a multi-domain cytochrome b_5 containing a molybdenum cofactor [75]. Electrons derived from sulfite are intramolecularly transferred through the heme cofactor to cytochrome *c*, while the oxygen atom is supplied by water, with concomitant production of sulfate (SO_4^{2-}) [75]. Overall, the mitochondrial SOU couples the oxidation of H_2S to thiosulfate and sulfate with the injection of electrons into coenzyme Q, leading to consumption of 0.79 O_2 per H_2S molecule oxidized, as reported by Goubern and co-workers [26].

Despite the apparent simplicity of the sulfide oxidation pathway herein described, the intricate sulfur chemistry and the remarkable substrate promiscuity of this pathway's enzymes allows one to envisage a number of alternative reaction pathways occurring under different (patho)physiological conditions. Irrespectively of the fate of sulfide-derived species, the major contribution of this pathway to cellular bioenergetics arises from the SQR-catalyzed reduction of coenzyme Q.

2.4. H_2S and cellular bioenergetics

A large variety of mammalian cell types (including colonocytes, macrophages, hepatocytes, neurons etc.) have been assayed for their ability to consume H_2S at the mitochondrial level [17,26–29], revealing a large variability of this sulfide oxidizing activity between cell types. The activity is high in cells physiologically exposed to relatively high H_2S levels, such as colonocytes, but absent or hardly detectable in other cell types, such as neuroblastoma or other nervous system-derived cell lines. Moreover, for yet unknown reasons, in mitochondria isolated from animal organs, the SOU activity relative to the basal respiratory oxygen consumption is typically much lower than that measured in intact cultured cells. As discussed in [8], this has been tentatively ascribed to an up-regulation of the SOU enzymes and/or to a down-regulation of the respiratory enzymes in cultured cells, typically relying on glycolytic metabolism.

Particularly interesting is the case of colonocytes. It has long been known that the intestinal microbiota, especially abundant in the colon, represents a major source of sulfide. Consistently, in this tract of our gut, sulfide reaches particularly high levels: up to 60 μM free H_2S was reported in equilibrium with up to one millimolar total sulfide-derived species [76–79]. It is therefore not surprising that colonocytes exhibit a high sulfide oxidizing activity, even at such high sulfide levels inhibitory for CcOX [17,26]. In these cells, when sulfide is oxidized, Complex I has been reported to work in a reverse mode, thus making oxidized coenzyme Q available for reduction by SQR, even in the presence of a severe CcOX inhibition [17,26]. Thus, colonocytes appear well adapted to the sulfide-rich colon environment: they are able to extract energy from a bacterial metabolic waste (H_2S) and are suitably equipped with an efficient dissimilatory system to prevent sulfide toxicity [76].

From the bioenergetic point of view and particularly in comparison with NADH oxidation, sulfide oxidation doesn't seem to be very efficient in terms of energy yield. This is not surprising as the oxidation of H_2S, while injecting electrons into coenzyme Q, is associated with the consumption of additional (with respect to the respiratory chain) O_2 by ETHE1 [16] (Fig. 1B). Despite this low energy yield, as discussed in [8], H_2S has several advantages, namely over NADH. It freely diffuses across

the membranes, without the need to be synthesized or transported inside mitochondria to feed the respiratory chain with electrons. In addition, H_2S is used with high affinity by SQR which ensures H_2S to be an effective respiratory 'fuel' at low, non-toxic concentrations. Last but not least, H_2S bioavailability inside the cell can be rapidly and finely controlled through regulation of the H_2S-synthesizing and -consuming enzymes (see below). Based on these properties, sulfide was suggested to serve as an 'emergency' substrate, particularly in some body regions and under specific physiological or stress conditions [8]. In this regard, it is intriguing that CGL, preferentially located in the cytosol, under cell dysfunction conditions translocates into the mitochondrion [80] and that under hypoxic conditions CBS does the same, exerting mitochondrial protective effects, as shown in cultured hepatocytes and liver tissue [81].

Several lines of evidence show that endogenously produced H_2S can contribute to cellular bioenergetics [29,80,82], but more work is needed in this direction. A bioenergetic role has been clearly demonstrated for MST [29]. Using cultured hepatoma cells and isolated rat liver mitochondria, low concentrations of the MST substrate, 3-MP, proved to lead to H_2S production and bioenergetic enhancement, and these effects proved to be mediated by MST and SQR by gene silencing [29]. In contrast, under normal conditions, at least in vascular smooth-muscle cells, CGL does not seem to support cellular bioenergetics. It is worth noticing, however, that upon intracellular calcium loading, the enzyme translocates to the mitochondrion where it exerts positive bioenergetic effects under hypoxic conditions [80]. Regarding CBS, an *in vivo* bioenergetic role was envisaged in *Caenorhabditis elegans*, whose basal and maximal carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP)-stimulated oxygen consumption was shown to be attenuated by silencing a CBS homologue, the main H_2S-producing enzyme in this model organism [83,84].

More recently, endogenous H_2S production by CBS was found to support cellular bioenergetics and cell proliferation in HCT116 colorectal cancer cells [82]. In these cells, CBS is selectively up-regulated (like in human colon adenocarcinoma specimens) and a significant fraction of the enzyme is associated to mitochondria. Moreover, as shown by CBS silencing or inhibition with aminooxyacetic acid (AOAA), the CBS-generated H_2S enhances cell proliferation and energy metabolism. Both oxidative phosphorylation and glycolysis proved to be stimulated by CBS-produced H_2S, the glycolytic pathway being presumably enhanced in response to persulfidation and consequent activation of glyceraldehyde 3-phosphate dehydrogenase [85,86]. Interestingly, AOAA, which has been reported to inhibit both CBS and CGL [87], decreased the migration and invasion of cancer cells, reduced the migration of endothelial cells co-cultured with colon cancer cells and, notably, affected the growth rate of both HCT116 and patient-derived tumour xenografts [82]. None of the effects described above was observed upon CGL silencing or pharmacological inhibition with the CGL-selective inhibitor propargylglycine, pointing to a specific role for CBS in colorectal cancer cell bioenergetics.

In summary, there is growing evidence that H_2S, depending on its bioavailability, can impact cellular bioenergetics by stimulating or inhibiting the mitochondrial respiratory chain and, under certain conditions, enhancing glycolysis. These functions of H_2S are interestingly intertwined with the action of the other two gasotransmitters CO and NO. Hence the relevance of shedding light into the molecular mechanisms controlling the cell bioavailability of H_2S and its cross-talk with the other gasotransmitters.

3. Gasotransmitters interplay at human cystathionine β-synthase

3.1. Cellular localization and structure of human CBS

CBS is a key enzyme of the transsulfuration branch of methionine metabolism, that plays a key role in mammalian physiology both contributing to homocysteine homeostasis and acting as a source of H_2S [13,88,89]. Whereas CBS is typically considered a cytosolic enzyme, its localization has been shown to vary in different (patho)physiological contexts. Microvascular endothelial cells and hepatocytes have been reported to secrete CBS and CGL, which circulate as part of the plasma proteome, generating H_2S from homocysteine, particularly when its concentration rises in human blood [90]. In endothelial cell models, exogenous H_2S afforded higher cell viability and decreased DNA oxidative damage upon serum starvation and hypoxia/reoxygenation. Moreover, immunoprecipitation of CBS and CGL from serum prior to its supplementation with homocysteine makes the serum more stressful for endothelial cells. These observations led to the proposal that secreted CBS and CGL ensure both the clearance of elevated homocysteine and the production of H_2S to exert a protective role in the endothelium [90]. CBS has also been shown to be a target for sumoylation, leading to accumulation of the protein in the nucleus, as associated with the nuclear scaffold [91]. Of greater relevance for the effect of H_2S on cell bioenergetics and respiration, CBS can translocate to mitochondria in different (patho)physiological conditions. Teng and co-workers [81] reported partial localization of CBS to liver mitochondria, transiently enhanced under hypoxia/ischemia to protect mitochondria from oxidative stress through H_2S generation. CBS levels are restored upon return to normoxia, the protein degradation being mediated by the mitochondrial Lon protease, targeting specifically the CBS heme-binding domain in the oxidized (ferric) form [81]. Moreover, in the HCT116 colorectal cancer cell line, a significant fraction of the total cellular CBS is localized to the mitochondria, particularly associated with the outer mitochondrial membrane, where it was proposed to stimulate mitochondrial functions [82], as described above. Increased accumulation of CBS concurrent with elevated H_2S has been recently reported in mitochondria isolated from the spinal cord of a mouse model of amyotrophic lateral sclerosis, where the increased H_2S production was proposed to be associated with impaired cytochrome *c* oxidase-dependent respiration [92].

Each CBS monomer comprises a PLP-binding core catalytic domain and two flanking regulatory domains: an N-terminal heme-binding domain and a C-terminal domain which binds the allosteric activator S-adenosyl-L-methionine (AdoMet) (Fig. 4) [93]. The PLP-binding catalytic core is characteristic of type II PLP-dependent enzymes [93,94]. The

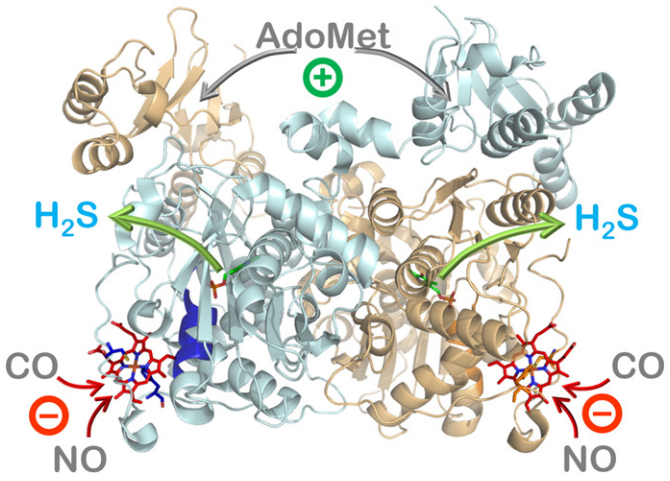

Fig. 4. Structure of human cystathionine β-synthase. Cartoon representation of the 'full-length' human CBS homodimer (PDB ID: 4COO; Δ516-525; 2.0 Å resolution), where the C-terminal domain of each monomer (coloured cyan or light orange) hinders the substrate entrance to the active site of the opposite monomer. AdoMet binding causes the C-terminal domains to associate in a disk-like form, de-repressing the enzymatic activity. Red sticks, regulatory heme *b* with its Cys_{52} and His_{65} ligands, where NO or CO binds, resulting in enzyme inhibition. Green sticks, active site PLP moiety, where H_2S production occurs. Bright blue and bright orange helices (residues T_{257}-G_{258}-G_{259}-T_{260}-I_{261}-T_{262}-G_{263}-I_{264}-A_{265}-R_{266}) communicate the heme redox and ligand binding state to the PLP active site. Figure generated with Pymol.

C-terminal domain, structurally also known as "Bateman" module, comprises a pair of so-called CBS motifs able to accommodate AdoMet [94–96]. This domain has been suggested to be also responsible for enzyme tetramerization, its removal resulting in stabilization of a dimeric form of CBS unresponsive to AdoMet and displaying increased basal activity [97].

3.2. Properties of the heme moiety of human CBS

The heme in CBS constitutes an evolutionary milestone, since its harbouring domain is absent in protein homologues from unicellular eukaryotes. This low-spin hexacoordinate B-type heme, axially bound to His65 imidazole and Cys52 thiolate moieties (human CBS numbering), has been proposed to act not only as a redox sensor able to bind exogenous ligands (CO and NO), but also to structurally stabilize CBS and improve its folding [97]. Whereas the enzyme is fully active when the heme is in the ferric state, heme reduction by excess sodium dithionite (a strong reductant) has been reported to promote a slow enzyme inactivation (>20 minutes at 37°C), attributed to a ligand switch process [98]. Notably, the CBS heme in the reduced state is able to bind the gasotransmitters NO and CO, resulting in a reversible enzyme inhibition (detailed below) [99–101]. The CBS heme has thus been considered a redox and ligand sensor able to modulate CBS activity (reviewed in [102]). The communication between the heme and PLP active site has been attributed to molecular interactions at either end of the α-helix 8 [103–105]. While at the heme end of this helix Arg266 establishes electrostatic interactions with the Cys52 thiolate, on the opposite end Thr257 and Thr260 are involved in a hydrogen-bond network with the PLP phosphate moiety (Fig. 4). The role of these residues in communicating the heme redox and ligand state to the PLP site has been confirmed by functional and spectroscopic studies on clinically relevant site-directed variants of Arg266, Thr257 and Thr260 [103–105]. Although the CBS heme has a low reduction potential (ca. -350 mV, [103]), in the presence of NADPH the human enzyme methionine synthase reductase (MSR) has been shown to be able to catalyze its reduction *in vitro* and generate both the ferrous-CO and the ferrous-NO adducts [106,107].

The UV–visible spectral changes associated with heme reduction and CO or NO binding to the reduced heme are depicted in Fig. 5A. Upon reduction, the Soret band of the oxidized heme centered at 428 nm is shifted to 449 nm (Fig. 5A), similarly to the heme thiolate-containing cytochromes P450. Upon CO binding to the ferrous CBS heme, the band shifts to 422 nm (Fig. 5A), resulting in a hexacoordinate ferrous-CO adduct with the endogenous Cys52 ligand displaced, as confirmed by Raman spectroscopy [108]. NO binding to ferrous CBS results instead in a notable broadening of the Soret band, accompanied by an intensity decrease and a blue shift to ~395 nm (Fig. 5A), which have been attributed to formation of a high-spin pentacoordinate ferrous-NO adduct with both endogenous ligands displaced, as confirmed by EPR spectroscopy [101]. Despite the inhibitory effect of CO and NO on CBS, the enzyme activity is restored upon reoxidation by O_2 or potassium ferricyanide, both converting the CO or NO ferrous adducts to the ferric state [99,101].

The kinetics of this interplay between the three gasotransmitters has been investigated by a combination of static and stopped-flow absorption spectroscopy [100,101,106,107,109–111].

3.3. Physiological relevance of CBS inhibition by NO and CO

The physiological relevance of CBS regulation by NO and CO has been attested by different lines of *in vivo* evidence. In mice experiencing renal ischemia-reperfusion, elevated NO levels have been shown to inhibit CBS, yielding an increase in homocysteine deleterious for the kidney [112]. Increased NO production by endothelial cells and macrophages during endotoxemia has been shown to result in mitochondrial dysfunction and elevated oxidative stress in adrenocortical cells, leading

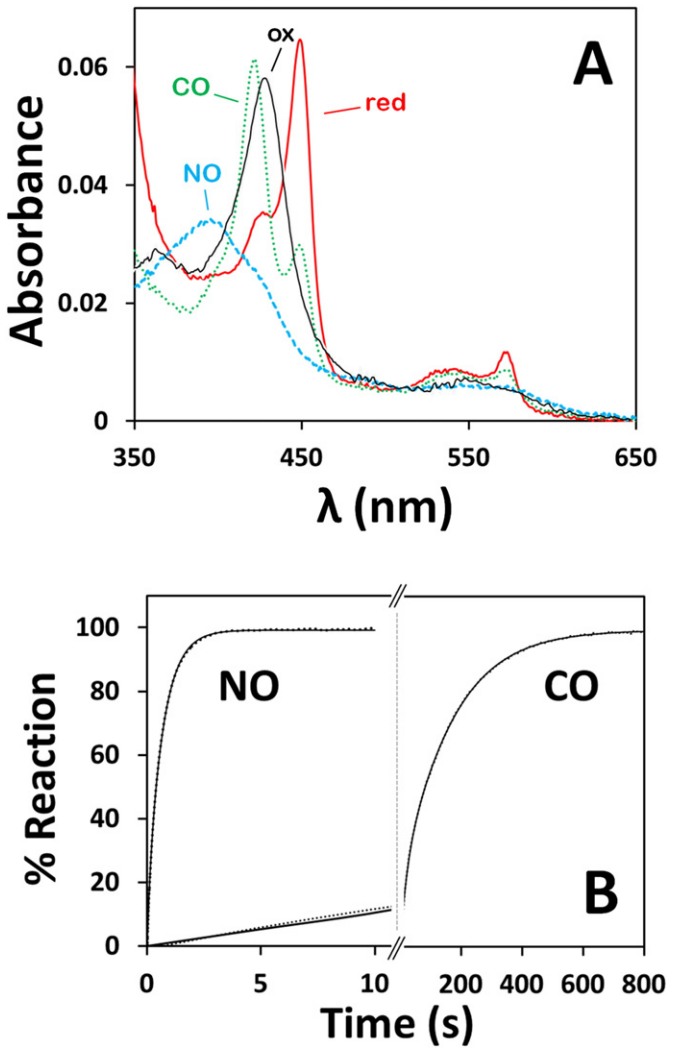

Fig. 5. Spectroscopic and kinetic analysis of NO and CO binding to human CBS. *Panel A*, UV–visible absorption spectra of human CBS (in 50 mM phosphate buffer, 300 mM KCl, 10% glycerol, pH 7.0) in the oxidized (black line), dithionite-reduced (red line), ferrous-CO bound (dotted green line) and ferrous-NO bound (dashed blue line) states. *Panel B*, kinetic traces acquired after stopped-flow mixing ferrous human CBS with NO (400 μM after mixing) or CO (500 μM after mixing) at 25 °C.
Adapted from [110].

to adrenal insufficiency, whereas the adverse effects of increased NO were suppressed upon supply of an exogenous H_2S donor [113]. It was proposed that NO-mediated inhibition of CBS contributes to mitochondrial dysfunction and impaired steroidogenesis in adrenal cortex during endotoxemia [113]. Cerebral vasodilation in a mouse model of hypoxia was reported to be associated with CO-mediated CBS regulation [114]. Under hypoxic conditions, the CO-producing oxygen sensor heme oxygenase HO-2 is down-regulated. The resulting decreased CO levels alleviate the CO inhibitory effect on CBS-catalyzed H_2S production, contributing to mediate vasodilation of precapillary arterioles [114]. Increased CO in the liver has been shown to cause a decrease in H_2S levels and a concomitant increase of bile output. These effects were observed in wild-type but not in heterozygous CBS knockout mice, leading to the proposal that CO inhibition of CBS-mediated H_2S production affords a control mechanism of bile excretion, at least in stressful and/or pathological conditions [115]. The relevance of CO inhibition of CBS activity has been attributed not only to its H_2S-generating ability, but also to its 'hinge' role between the remethylation and the transsulfuration pathways (reviewed in [116]). CBS inhibition by CO diverts

homocysteine to the remethylation pathway (Fig. 1A), making AdoMet more available for protein and nucleic acid methylation. On the other hand, an opposing effect produced by CBS inhibition arises from the accumulation of S-adenosyl-L-homocysteine (AdoHcy), an inhibitor of specific methyltransferases. This imbalance caused by CO inhibition of CBS has been shown to affect the global protein methylation status (e.g. causing hyper-methylation of histone H3), leading to the hypothesis that CBS inhibition by CO plays a role in the regulation of cancer cell survival [116,117].

3.3.1. CO binding to human CBS

In the past few years, several groups (including ours) have thoroughly investigated *in vitro* CO and NO binding to the heme moiety of human CBS, to bring together the data acquired on the isolated enzyme with the *in vivo* data supporting the physiological relevance of this regulatory mechanism. CO binding to ferrous CBS is characterized by two dissociation constants, $K_{d1} = 0.7–1.5$ μM and $K_{d2} = 45–68$ μM [100,110,111], attributed to differences in the heme microenvironment [100] and/or an anti-cooperative effect between adjacent monomers within a functional CBS dimer [108]. Overall CO binding has been reported to proceed with a C_{50} value of 29 ± 4 μM [111] and >30 μM (estimated from Fig. 2 in [100]). The kinetics of CO binding to ferrous CBS was investigated by time-resolved spectroscopy [108,110,111]. Upon mixing ferrous CBS with CO solutions, formation of the ferrous-CO adduct occurs without optical evidence for reaction intermediates, as judged by global fit analysis. The reaction time course is at least biphasic (Fig. 5B), with a major slow phase the rate constant of which is hyperbolically dependent on CO concentration, yielding a limiting value $k_{lim} = 0.012–0.017$ s^{-1} [108,110]. As confirmed by laser flash photolysis time-resolved Raman spectroscopy, this rate constant has been attributed to the slow rate-limting dissociation of the endogenous Cys52 thiolate ligand from the ferrous heme iron [108]. The k_{off} of CO was found to be 0.5 ± 0.1 s^{-1} in stopped-flow displacement experiments carried out mixing the ferrous-CO adduct with an excess (0.1–1.9 mM, before mixing) of authentic NO [110].

3.3.2. NO is a quick and tight ligand of human CBS

NO binding to the reduced heme of human CBS has been initially reported to be characterized by a very low affinity (K_d value of 281 ± 50 μM), incompatible with a physiological relevance of this reaction [101]. In that study, however, the K_d for NO was likely overestimated because measurements were carried out using a slow NO releaser (diethylamine NONOate) in the presence of large excess of dithionite, which is known to promptly react with free NO. Using a different reduction system, involving NADPH and human MSR, a ~10-fold lower K_d value was reported (30 ± 5 μM), still considered an upper limit for this dissociation constant, as it was measured in the presence of multiple equilibria (NADPH ↔ MSR ↔ CBS ↔ CBS-NO) [107]. More recently, employing authentic NO stock solutions and low dithionite concentrations to reduce CBS, a $K_d \leq 0.23$ μM was measured [110], fully compatible with a physiological role of this molecular interaction. Reduced human CBS, therefore, displays a much (>100-fold) higher affinity for NO than for CO.

Interestingly, NO binds ferrous CBS much more quickly than CO (Fig. 5B). The kinetics of NO binding to ferrous heme in human CBS was analyzed by stopped-flow absorption spectroscopy [110]. No reaction intermediates were detected during the conversion of hexacoordinate ferrous CBS into the pentacoordinate ferrous-NO species. Overall, the reaction of reduced CBS with NO was significantly faster than with CO. As shown in Fig. 5B, 500 μM CO requires ~10 min for conversion of ferrous CBS into the ferrous-CO adduct, whereas formation of the ferrous-NO adduct is complete within ~3 s after mixing ferrous CBS with 400 μM NO. Also differently from CO, the observed association rate constant for NO is linearly dependent on [NO] up to ~800 μM, yielding $k_{bim} = 8 \times 10^3$ M^{-1} s^{-1} [110]. NO dissociation kinetics was analyzed by the Moore and Gibson method [118], by mixing ferrous-NO CBS

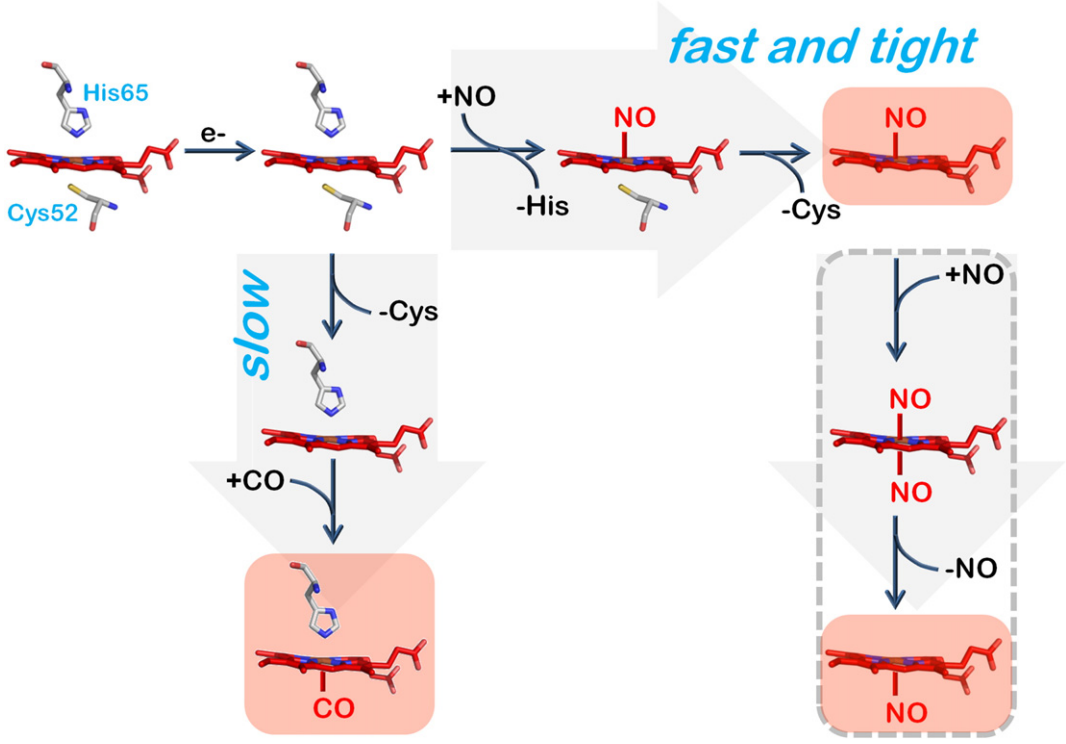

Fig. 6. Proposed mechanisms for NO and CO binding to human CBS. Whereas CO binding to ferrous CBS appears to be rate-limited by the displacement of Cys52, NO is proposed to displace first the His65 ligand and end-up as a pentacoordinate ferrous-NO adduct with both endogenous axial ligands displaced and NO laying on either the His or the Cys side. In the latter case, an obligate hexacoordinate ferrous-dinitrosyl (NO-Fe-NO) species should form transiently. As there is no direct evidence for the occurrence of such a dinitrosyl intermediate in the reaction of CBS with NO, these yet hypothetical reactions are depicted inside a dashed box.

with a CO-saturated 200 mM dithionite solution. In accordance with the high affinity for NO, dissociation of this ligand occurred slowly, with a $k_{obs} = 0.003\ s^{-1}$ more than 100-fold slower than the CO off rate constant ($0.5 \pm 0.1\ s^{-1}$) [110].

Thus, NO binds to ferrous CBS at rates orders of magnitude higher than those observed for CO binding and it dissociates from the ferrous heme iron much more slowly than CO. Altogether, these data neatly fit with the much higher affinity for NO with respect to CO. From a mechanistic viewpoint, the fact that kinetics of NO binding is not limited by the off-rate of the Cys52 ligand was taken as the evidence that NO initially attacks the Fe from the His65 side, rather than from the Cys52 side [110] (Fig. 6). Moreover, since that reaction yields a pentacoordinate ferrous-NO adduct as the final product [101] with no evidence for a transiently generated hexacoordinate Cys-Fe-NO adduct, NO binding to the His side was suggested to cause a concomitant displacement of Cys52, at rates much higher than its intrinsic off rate (that instead limits CO binding). It remains to be established whether the NO bound in the final pentacoordinate adduct lays on the His or the Cys side. For NO to end up on the Cys side, an obligate hexacoordinate ferrous-dinitrosyl (NO-Fe-NO) species should form transiently during the reaction, as observed with other hexacoordinate heme proteins (e.g. bacterial cytochrome *c*' and heme NO/O_2 sensor, and soluble guanylate cyclase) where high affinity for NO is attained by sequential NO binding steps preceding formation of the final pentacoordinate NO-bound species [119–123]. However, direct spectroscopic evidence for the formation of such a hexacoordinate ferrous-dinitrosyl intermediate during the reaction of CBS with NO is lacking. For this reason, these yet hypothetical reactions are depicted inside a dashed box in Fig. 6. Irrespectively of the NO binding mechanism, it remains to be understood why in human CBS NO initially binds to the His side, whereas CO to the Cys side. In bacterial cytochrome *c*′, binding of NO and CO on opposite sides of the heme has also been reported [121,124–126]. The affinity for either gas in heme proteins has been assigned to the chemical nature of the endogenous iron axial ligands and to the properties of the heme pocket environment, as a cumulative effect of steric hindrance, electrostatic interactions and H-bonds [119,120,123].

3.3.3. Effect of S-adenosyl-L-methionine on CO and NO inhibition of human CBS

As a follow-up of the investigations described above, a thorough analysis of the effect of the allosteric activator AdoMet on the heme chemistry of human CBS has been recently undertaken, focusing on the CO and NO binding properties of the enzyme [111]. Structural studies have elucidated the molecular basis of AdoMet stimulation of CBS activity [94,96]. In the AdoMet-free state, the C-terminal domain from one monomer hinders substrate accessibility to the active site of the adjacent monomer. Upon AdoMet binding, a large conformational change takes place and the C-terminal domains from the two monomers associate in a disk-like shape, thereby unblocking the entry to the substrate pocket and facilitating its access to the PLP active site.

Despite the significant distance between the C-terminal AdoMet-binding and the N-terminal heme-binding domains (>30 Å between adjacent monomers and >50 Å within one monomer in AdoMet-free CBS), the question was raised as to whether AdoMet binding at the C-terminus promotes a distal communication with the heme site [111]. Indeed, by analyzing H_2S production by reduced CBS as a function of CO concentration in the absence or presence of AdoMet, we estimated a $K_i = 9.5 \pm 1.0$ μM for AdoMet-free CBS and a $K_i = 0.7 \pm 0.1$ μM for the AdoMet-bound enzyme (Fig. 7A) [111]. These data not only provided the first report of a K_i for CO regarding CBS-catalyzed H_2S production, but also revealed a marked functional effect of AdoMet on the heme-mediated CO inhibition of the enzyme.

Prompted by these observations, a thorough characterization of the CO and NO binding properties of human CBS in the presence or absence of AdoMet has been undertaken [111]. As shown in Fig. 7B and in line

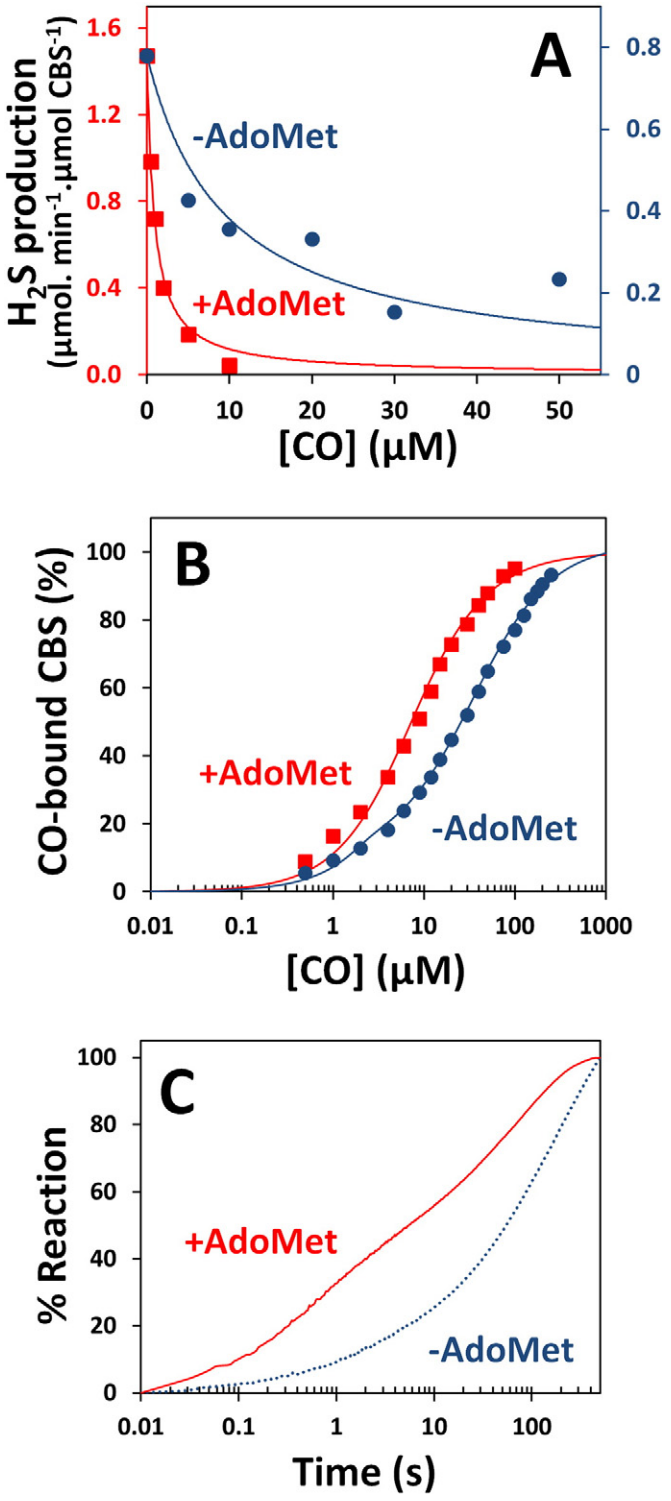

Fig. 7. AdoMet enhances CO binding to human CBS and inhibition of H_2S production. *Panel A*, CO inhibition of H_2S production by human CBS in the absence or presence of S-adenosyl-L-methionine (AdoMet), assayed at 37 °C. Data fitting yielded $K_i = 9.5 \pm 1.0$ μM and $K_i = 0.7 \pm 0.1$ μM in the absence and presence of AdoMet, respectively. *Panel B*, CO titration profiles obtained by global fit of the UV–visible spectral data acquired in the absence or presence of 500 μM AdoMet. Data fitting yielded $C_{50} = 6 \pm 1$ μM and $C_{50} = 29 \pm 4$ μM for AdoMet-bound and AdoMet-free CBS, respectively. *Panel C*, reaction time courses obtained by stopped-flow mixing ferrous human CBS with 1 mM CO in the absence or presence of AdoMet (500 μM before mixing). Data fitting yielded $t_{1/2} = 5.5$ s and $t_{1/2} = 55$ s for AdoMet-bound and AdoMet-free CBS, respectively. Reaction conditions and data analysis detailed in [111].

with the effect of AdoMet on CO inhibition, by performing anaerobic CO titrations of reduced CBS monitored by UV–visible absorption spectroscopy, AdoMet proved to significantly increase the affinity of the enzyme for CO. Particularly, AdoMet binding resulted in an overall ~5-fold decrease of the C_{50} value, from 29 ± 4 μM to 6 ± 1 μM. Carrying out control titrations with a truncated CBS form lacking the C-terminal AdoMet-binding domain, a C_{50} of 12 ± 1 μM was estimated both in the absence and presence of AdoMet, ruling out an unspecific direct effect of AdoMet on the heme [111].

The effect of AdoMet on CO binding kinetics was then evaluated by stopped-flow mixing AdoMet-free or AdoMet-bound ferrous CBS with CO solutions. In accordance with the increased affinity measured in the presence of AdoMet, CO association to AdoMet-bound CBS was markedly faster than to the AdoMet-free enzyme (Fig. 7C) [111]. Indeed, incubation with AdoMet resulted in an overall ~10-fold faster association ($t_{1/2}$ = 5.5 s and $t_{1/2}$ = 55 s for AdoMet-bound and AdoMet-free CBS, respectively). The effect of AdoMet on CO binding was then analyzed at saturating CO concentrations and varying the AdoMet concentration [111]. The reaction followed a characteristic biphasic time course and both CO binding rate constants showed a hyperbolic dependence on AdoMet concentration, characterized by a C_{50} value of 12 ± 4 μM and 18 ± 3 μM for the fast and slow kinetic phase, respectively, showing a nearly saturating effect at physiological AdoMet concentrations (50–80 μM, [127]). Altogether, the acquired data show a significant functional impact of AdoMet on CO binding to ferrous human CBS resulting in an increased propensity towards CO inhibition of H_2S synthesis.

The effect of AdoMet was further evaluated on the binding affinity and association kinetics of NO [111]. As compared to CO, AdoMet exhibited a less pronounced effect on NO binding. Affinity for NO increased by ~2-fold in the presence of AdoMet, consistent with a ~1.5-fold increase in the association bimolecular rate constant and ~1.3-fold decrease in the dissociation observed rate constant [111]. Notably, the association observed rate constants at a fixed NO concentration displayed a hyperbolic dependence on AdoMet concentration, yielding a C_{50} of 16 ± 2 μM for AdoMet, fully consistent with those determined for CO and with a modulation of NO binding to CBS at physiological AdoMet concentrations. As with CO, a truncated version of CBS lacking the C-terminal AdoMet-binding domain proved to be insensitive to AdoMet both in terms of NO binding affinity and kinetics [111].

The fact that AdoMet affects differently CO and NO binding is fully consistent with the proposal that NO and CO attack initially the CBS heme iron from different sides (Fig. 6). Since AdoMet affects more pronouncedly CO binding, it is likely that AdoMet binding at the C-terminal regulatory domain results into a selective weakening of Fe-Cys52 bond. It remains to be determined whether this functional communication between distant regulatory sites within CBS also involves the catalytic core domain. Irrespectively of the mechanistic details entailing this functional and structural communication between the CBS domains, it is remarkable that an allosteric effector that increases the enzyme activity, at the same time makes the enzyme more prone to inhibition by the exogenous ligands CO and NO. Despite this seemingly paradoxical regulatory conundrum, it is not surprising that CBS, being a major source of H_2S, exhibits such intricate switching mechanisms to quickly, effectively and reversibly modulate H_2S production, although the reflection on bioenergetics of the gasotransmitters interplay at the level of CBS remains to be elucidated.

In conclusion, growing evidence suggests that endogenous H_2S can impact cellular bioenergetics by modulating (either positively or negatively) mitochondrial respiration and, under certain conditions, enhancing glycolysis. Depending on its concentration, H_2S can either act as an effective substrate or, similarly to CO and NO, as an inhibitor of the mitochondrial respiratory chain, targeting CcOX. This dual function of H_2S, while crucially depending on the bioavailability of this gaseous signalling molecule, is interestingly intertwined with the action of the other two gasotransmitters CO and NO. It is therefore important to increase our knowledge on the molecular mechanisms underlying H_2S metabolism, its regulation and the intricate cross-talk between the three gasotransmitters, all contributing to a correct balance between H_2S synthesis and breakdown. In this light, a key role could be played by human CBS, an enzyme sitting at the crossroad between the signalling pathways of the three gasotransmitters. The field is in its infancy and clearly deserves in the future more efforts to answer the many as yet open questions.

Transparency document

The Transparency document associated with this article can be found, in the online version.

Acknowledgments

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