Sulfide inhibition of and metabolism by cytochrome c oxidase

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Abstract
Hydrogen sulfide (H₂S), a classic cytochrome c oxidase inhibitor, is also an in vitro oxidase substrate and an in vivo candidate hormonal (‘gasotransmitter’) species affecting sleep and hibernation. H₂S, nitric oxide (NO) and carbon monoxide (CO) share some common features. All are low-molecular-mass physiological effectors and also oxidase inhibitors, capable of binding more than one enzyme site, and each is an oxidizable ‘substrate’. The oxidase oxidizes CO to CO₂, NO to nitrite and sulfide to probable persulfide species. Mitochondrial cytochrome c oxidase in an aerobic steady state with ascorbate and cytochrome c is rapidly inhibited by sulfide in a biphasic manner. At least two successive inhibited species are involved, probably partially reduced. The oxidized enzyme, in the absence of turnover, occurs in at least two forms: the ‘pulsed’ and ‘resting’ states. The pulsed form reacts aerobically with sulfide to form two intermediates, ‘P’ and ‘F’, otherwise involved in the reaction of oxygen with reduced enzyme. Sulfide can directly reduce the oxygen-reactive ΔG⁻CuB binuclear centre in the pulsed state. The resting enzyme does not undergo such a step, but only a very slow one-electron reduction of the electron-transferring haem a. In final reactivation phases, both the steady-state inhibition of catalysis and the accumulation of P and F states are reversed by slow sulfide oxidation. A model for this complex reaction pattern is presented.

Introduction
Hydrogen sulfide (H₂S) is as poisonous as hydrogen cyanide (HCN) and almost as volatile; its lethal character has long been recognized (see, e.g., [1]). Since the identification of the terminal oxidase as a haem enzyme (cytochrome c oxidase or cytochrome aa₃), this behaviour has been recognized as being due to binding of ferric haem [2]. The use of sulfide thus creates individual risks, especially at the levels used industrially. One example is the large amounts of sulfide required for the preparation of deuterium oxide by the Girdler process [3], used in the development of the ‘CANDU’ heavy water reactor by, among others, Atomic Energy Canada. This led to research identifying sulfide as an in vitro oxidase substrate as well as inhibitor [4,5]. More recently, sulfide has been shown to be in vivo a hormone-like or ‘gasotransmitter’ species affecting vasodilation, sleep and hibernation [6,7]. It has consequently been identified as a potential chemotherapeutic agent [8]. However, the mechanism of these beneficial effects, and whether they bear any relationship to the oxidase inhibition that is likely to be responsible for the environmental toxicity, remains unclear [9].

Sulfide, nitric oxide (NO) and carbon monoxide (CO) share some characteristic features. All are low-molecular-mass but possible physiologically active species [6], they are also oxidase inhibitors [9,10], capable of binding at more than one enzyme site, and each is an oxidizable ‘substrate’. The oxidase oxidizes CO to CO₂, NO to nitrite and H₂S to one or more sulfane or persulfide species.

Mitochondrial oxidase is thus both a possible in vivo target for endogenously formed sulfide and a sink for its removal when biosynthesis ends. How are these two functions combined mechanistically?

Cytochrome c oxidase sulfide inhibition
The inhibition of active cytochrome c oxidase can be monitored continuously by measuring the steady-state reduction of the immediate electron donor cytochrome c by a reductant such as ascorbate (Figure 1). The rate of electron transfer in solution is proportional to the concentration of oxidized cytochrome c, and the rate of oxidase turnover is proportional to the concentration of reduced cytochrome c under so-called ‘Smith–Conrad’ conditions [4,11,12]. The fractional oxidase activity is therefore given by eqn (1) (see Figure 1 legend):

\[
[E]_{\text{experimental}} = \left[ c^{2+} \right]_0 \times \left[ c^{3+} \right]_t / \left[ c^{3+} \right]_0 \times \left[ c^{2+} \right]_t...
\]

Figure 1 (inset) plots the resulting time course values in a typical case and the theoretical fit to the double exponential equation (eqn 2), where \( t \) is the time after sulfide addition (see Figure 2 legend):

\[
[E]_{\text{simulation}} = 0.81e^{-0.24t} + 0.15e^{-0.055t} + 0.04...
\]

The initial inactivation rate is proportional to total sulfide concentration, with an initial rate constant at pH 7.4,
Cytochrome c oxidase: sulfide inhibition at steady-state turnover

Conditions: 0.1 M sodium phosphate (pH 7.47), 0.018% dodecyl maltoside, 30°C, 16 μM horse heart cytochrome c; 0.24 μM beef heart cytochrome aa3 (last form, prepared by modification [20] of the original Yonetani [21] procedure), with 14.5 mM sodium ascorbate, 11 μM Na2S and 180 μM TMPD (N,N,N',N'-tetramethyl-p-phenylenediamine) were added as indicated. The inset shows the fraction of active enzyme E with time after sulfide addition calculated as $[E] = [c^{2+}]_0 \times [c^{1+}]_0 / [c^{1+}]_0 \times [c^{1+}]_0$, where $[c^{2+}]_0$ = fraction ferrocytochrome c at zero time, $[c^{1+}]_0$ = fraction ferricytochrome c at time 0, $[c^{1+}]_0$ = fraction ferrocytochrome c at zero time, and $[c^{2+}]_0$ = fraction ferrocytochrome c at time 0. Symbols indicate experimental data; continuous line indicates double exponential fit. $[E]$ (simulation) = 0.81×exp(−0.24t) + 0.15×exp(−0.055t) + 0.04, where $k_0 = 2.2 \times 10^{10} \text{M}^{-1}\text{s}^{-1} (81\%)$, $k_5 = 5 \times 10^3 \text{M}^{-1}\text{s}^{-1} (15\%)$ and 4% final active form at 11 μM Na2S gives a $K_i$ value of 0.45 μM. Abs, absorbance.

$k_{on} = 2.2 \times 10^4 \text{M}^{-1}\text{s}^{-1}$. This is almost 10-fold greater than the corresponding cyanide rate. The final residual activity corresponds to an apparent inhibition constant, $K_i$, equal to 0.45 μM.

The complexity of the inhibition kinetics can be attributed to the occurrence of more than one step in sulfide binding, as in the case of cyanide [11], and summarized in eqns (3A) and (3B):

\[
a^{1+}_{3} \text{CuB}^{2+} (\text{H}_2\text{S}) \rightarrow a^{1+}_{3} \text{CuB}^{2+} \text{SH}^- = \]
\[
a^{1+}_{3} \text{SH}^- \text{CuB}^{2+} (+e^-) \rightarrow a^{3+}_{3} \text{SH}^- \text{CuB}^+ \ldots \quad (3A)
\]

\[
a^{1+}_{3} \text{CuB}^{2+} (+e^-) \rightarrow a^{1+}_{3} \text{CuB}^+ (+\text{H}_2\text{S}) \rightarrow \]
\[
a^{1+}_{3} \text{CuB}^+ \text{SH}^- = a^{3+}_{3} \text{SH}^- \text{CuB}^+ \ldots \quad (3B)
\]

Such a reaction sequence, with multiple oxidized and mixed valence forms of the oxidase interacting with sulfide, is also required to explain the non-competitive kinetics of sulfide inhibition with respect to oxygen concentration obtained by Petersen [13]. If the catalytic cycle with oxygen involved only fully oxidized and reduced states of the binuclear center, then inhibitors that bound primarily only one of the two states would exhibit competitive (e.g. CO, NO) or uncompetitive (e.g. HCN, H2S) behaviour. H2S, unlike HCN, NO and CO, has no binding capability for ferrous haem $a_3$. The simplest model then requires uncompetitive behaviour with respect to oxygen concentration. Non-competitive kinetics imply the occurrence of a sulfide-interacting species whose relative concentration increases as oxygen partial pressure decreases. The mixed valence state of the binuclear centre may represent such a species. It also provides the redox state seen in the final fully inhibited form of the enzyme.

The final sulfide-inhibited form of cytochrome c oxidase

Both ferric haem $a_3$ and CuB (either cuprous or cupric) can bind H2S (or SH−). The final oxidase product of sulfide inhibition in reactions such as that illustrated in Figure 1 is a mixed valence form of the enzyme in which haem $a$ and CuA are reduced and haem $a_3$ is in a ferric sulfide-bound state [12,14] as in eqn (3). This state is reached either by sulfide binding followed by reduction (eqn 3A) or vice versa (eqn 3B). Nicholls et al. [12,14] observed that the resulting $a$ absorbance maximum of ferrous cytochrome $a$ is blue-shifted, as earlier observed for the mixed valence azide complex. Later, Nicholls and Hildebrandt [15] showed that alkyl sulfides, also oxidase inhibitors, produced a much greater blue shift, similar to that achieved with azide.
Figure 2 | Reactions of resting and pulsed enzyme with sulfide

(A) Time courses of sulfide reaction with cytochrome c oxidase. Resting (‘slow’) enzyme prepared by modified Yonetani procedure [20,21]. Fast (‘pulsed’) enzyme prepared at high pH (8.0) with reductant present. Soret band red shift monitored at 433–431 nm (‘spin state’ changes with time). Conditions: 2.5 μM oxidase, 0.1 M sodium phosphate (pH 7.4), 0.05% dodecyl maltoside, 7 μM sulfide, 26.5°C. Final additions of 7 mM ascorbate and 180 μM TMPD (N,N,N’,N’-tetramethyl-p-phenylenediamine) were as indicated. (B) Cytochrome aa₃: initial and final sulfide difference spectra. Resting (‘slow’) enzyme was prepared by modified Yonetani procedure [20,21]. Fast (‘pulsed’) enzyme was prepared at high pH (8.0) with reductant present. Continuous lines show the early sulfide effect (20 s), producing a compound P species in the fast enzyme. Broken lines show later effect (90 or 200 s) and compound F species in the fast enzyme. Other conditions as in (A). abs, absorbance.

Bulky ligands such as azide and the alkyl sulfides at the haem a₃ iron centre thus exert a conformational effect across the transmembrane helix containing the fifth coordination position histidine ligands of both haems. The blue shifts suggest an increase in hydrophobicity of the pocket containing haem a.

The state of the fourth metal centre, CuB, in the final inhibited enzyme remained uncertain until parallel EPR experiments were carried out by Hill et al. [16]. These workers showed the appearance of characteristic low-spin (g = 2.53, 2.23 and 1.86) signals of haem a₅, no longer coupled to cupric CuB. CuB remained reduced even upon reoxidation of the sulfide-bound enzyme. The oxidized sulfide-inhibited enzyme then showed EPR signals from three of the four metal centres, haem a₃, haem a and CuA. For CuB to remain cuprous in the presence of high oxidant levels implied a substantial increase in its redox potential in the sulfide-bound state, possibly due to ligation of CuB⁺ by a second sulfide ion, as in eqn (4):

$$a_3^{3+} \cdot SH^− \cdot CuB^{+}(+H_2S) \rightarrow a_3^{3+} \cdot SH^− \cdot CuB^+ \cdot SH^- \ldots \ldots (4)$$
The sulfide-metabolized form of cytochrome c oxidase

'As prepared' purified cytochrome c oxidase (see Moody [17]) contains a variable contribution from a slow 'resting' form of the enzyme, where the haem $a_3$ reacts sluggishly with external ligands. A cycle of reduction and reoxidation ('pulsing') can convert this into a fast form that reacts more rapidly with ligands [17]. When this fast form of the oxidized enzyme reacts with low sulfide levels and in the absence of reduced substrates, the initial product is not the inhibited enzyme, but instead a 'P' species characteristic of the immediate product of oxygen reaction with partially reduced ('mixed valence') enzyme [17,18]. This then decays into an 'F' species [18,19].

Figure 2 compares the reduction patterns of the fast and slow enzyme binuclear centres, with Figure 2(A) showing the time courses of reduction and Figure 2(B) showing the corresponding spectra. The initial rate at which the 607 nm ('P') species forms from the fast enzyme is similar to the rate of inhibition under steady-state catalytic conditions (Figure 1). Over a period of tens of seconds, the P form changes into a ferryl ('F') form by autoreduction or reduction by residual sulfide. In contrast, the resting enzyme shows almost no changes at the binuclear centre, but a partial reduction of haem $a$.

Eqsns (5A) and (5B) summarize the presumed reactions involved. Whether compound P formation requires one or two molecules of sulfide and whether the sulfide oxidation product is indeed hydrogen persulfide (H$_2$S$_2$) or a form of 'atomic' sulfur remains uncertain. Stoichiometric studies [5] do indicate that the uptake of oxygen in the initial process involves more than one sulfide equivalent.

$$a_3^{3+}\text{CuB}^{2+}(+\text{H}_2\text{S}) \rightarrow a_3^{3+}\text{CuB}^{2+}\text{SH}^- (+\text{H}_2\text{S}) \rightarrow$$
$$a_3^{3+}\text{SH}^-\text{CuB}^{2+}\text{SH}^- \rightarrow a_3^{3+}\text{CuB}^+ + \text{HSSH}... \quad (5A)$$

$$a_3^{2+}\text{CuB}^+(+\text{O}_2) \rightarrow \text{compound P (+e}^-\text{)} \rightarrow \text{compound}$$
$$\text{F(a}_3\text{O}^{2+}\text{CuB}^{2+})(+\text{e}^-) \rightarrow a_3^{3+}\text{CuB}^{2+}... \quad (5B)$$

Conclusions: an integrated scheme

In the cases of CO and NO, recognized gasotransmitters that are also oxidase substrates and inhibitors, the inhibited oxidase species and the oxidase species responsible for metabolism are different. CO and NO both bind primarily to the binuclear centre in the fully reduced (ferrous/cuprous) state, whereas they are oxidized (to CO$_2$ or nitrite) by resting or pulsed enzyme in the fully oxidized state. Sulfide does not seem to react with fully reduced enzyme and therefore both inhibitory and metabolic reactions with sulfide occur in the oxidized or mixed valence states.

Effective sulfide reduction of the binuclear centre to give compound P in an aerobic system requires the enzyme to be initially in a ‘fast’ or pulsed state (Figure 2). Resterizing enzyme accepts some electrons from added sulfide, partly inducing reduction of haem $a$, but forms very little doubly reduced binuclear centre (Figure 2). Inhibition by sulfide is a complex process (Figure 1 and associated legend), but presumably characteristic of the ‘pulsed’ state as this is automatically generated after a few turnovers in a catalytically active system. An integrated pattern of cytochrome c oxidase–sulfide reactions, combining the processes in eqns (3), (4) and (5), might therefore look like the scheme of Figure 3.

Not all the reactions shown in Figure 3 have been demonstrated individually by experiment. They are proposed for indirect (kinetic or other) reasons and so are included for completeness. Free sulfur ($S^0$ or $S_2^-$), sulfur radicals, persulfides and polysulfides may occur as reaction products and remain to be identified. Stopped-flow experiments are also being attempted to cover the great time range (microsecond to tens of minutes) over which relevant enzyme–sulfide interactions can occur ([10], and D.C. Marshall, C.E. Cooper and M.T. Wilson, unpublished work). Whether the oxidase (or indeed the mitochondrial sulfide-quinone reductase) provides a major dissimilatory mechanism for the endogenously generated gasotransmitter sulfide is uncertain. It is also not clear whether naturally occurring levels of sulfide, unlike those of NO [10], are sufficient to affect oxidase as a possible adaptive cellular target molecule. We await developments.

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