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OXIDATION OF SULPHIDE BY CYTOCHROME aa_3*

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The effectiveness of H_2S as an inhibitor of cytochrome *c* oxidase increase (K_i decreases) with sulphide concentration. A spectroscopic change in cytochrome aa_3 is induced aerobically by sulphide at the same rate as that calculated for inhibition. The initial spectroscopic product is not inhibited, but an 'oxygenated' (oxyferri) form of the enzyme. Stoichiometric sulphide addition to cytochrome aa_3 under anaerobic conditions produces another low-spin form of the enzyme; subsequent admission of oxygen gives rise to the 607 nm compound. At high enzyme levels sulphide itself acts as a substrate measured polarographically, with an oxygen uptake proportional to the amount of sulphide added. Binding of sulphide to ferric enzyme probably causes reduction at the oxygen-sensitive a_3-Cu centre, which is followed aerobically by reoxidation to the oxyferri state via the 607 nm intermediate. A stable sulphide complex is formed only after the reduction of cytochrome *a*; but once formed this inhibited species is retained if cytochrome *a* is reoxidized.

Sulphide, introduced as terminal inhibitor and haem ligand by Keilin [1], was later used quite extensively by Chance and Schoener [2] as a ligand for cytochrome a_3 that did not show the kinetic anomalies seen with cyanide or the spectroscopic anomalies found with azide. Later some puzzling features began to emerge. In the half-reduced sulphide-inhibited state, cytochrome $a^{2+}a_3{}^{3+}H_2S$, an unusually high Soret peak together with a small blue shift of the α-peak was seen [3], which seemed to represent an effect similar to that of azide and seen more clearly with alkyl sulphides [4]. In this state, but not in the oxidized enzyme, the presence of sulphide is associated with the appearance of a low-spin iron EPR signal at *g* 2.54, with [5] or without [6] a shoulder or auxiliary peak at *g* 2.56. A third low-spin signal can also be seen, apparently a precursor of either or both final signals, at *g* 2.77, on adding sulphide rapidly to anaerobically reoxidized enzyme [7]. A free radical (*g* approx. 2) signal can also been seen on adding sulphide to the enzyme aerobically [8].

Inhibition of the enzyme by sulphide takes place with a K_i of approx. 0.2 μM [3,9]. It appears to be non-competitive with respect to both substrates, cytochrome *c* and oxygen [9]. Like the inhibition by cyanide and by formate, the sulphide effect is not instantaneous. A finite time is required for the inhibited state to supervene [3]. Similarly, at low sulphide concentrations, the spectroscopic effects are also not immediate. In view of the several EPR-detectable sulphide-inhibited species [5–8], and the spectroscopic peculiarities in both half-reduced [3,4] and fully oxidized enzyme [10], it seemed worthwhile to compare the kinetics of spectroscopic change with those of inhibition, as attempted previously with cyanide [11].

Methods and Materials

Cytochrome aa_3 was prepared from beef heart according to the method of Kuboyama et al. [13], and stored at −75°C in 100 mM sodium and potas-

* A version of this paper has been presented at the 72nd annual (ASVC meeting in St. Louis, MO, U.S.A. [12].

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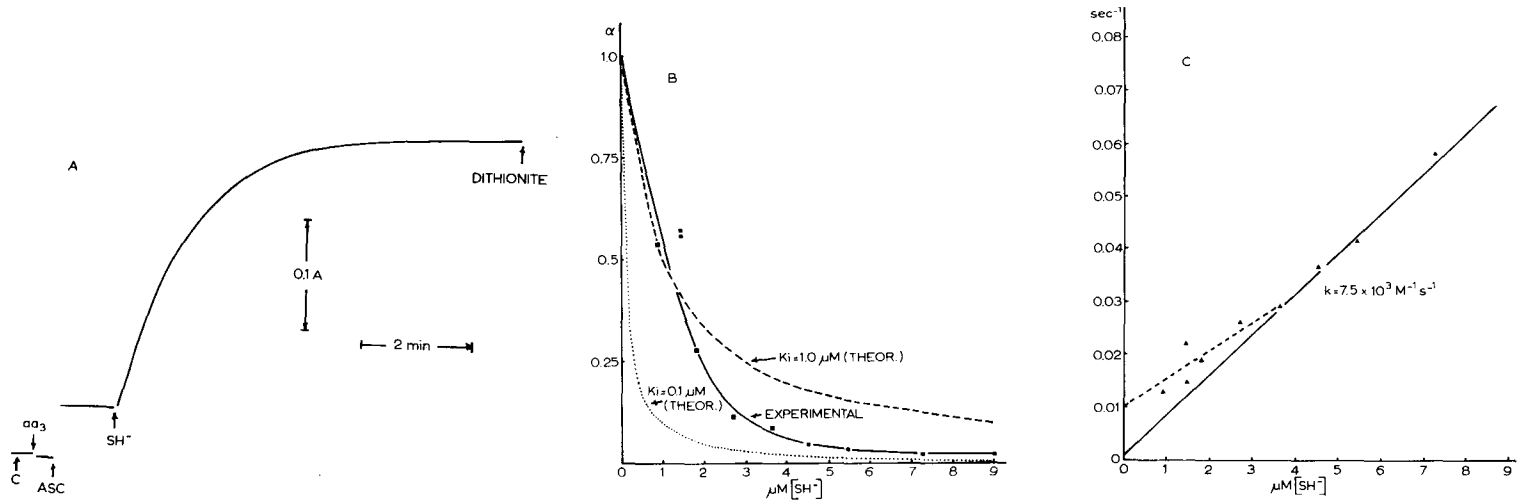


Fig. 1. Inhibition of cytochrome aa_3 by sulphide. (A) Steady-state reduction of cytochrome *c* in the presence of enzyme, ascorbate and inhibitor. 1.1 mM ascorbate (ASC) and 0.18 μM cytochrome aa_3 (aa_3) were added to 18.5 μM cytochrome *c* (*c*) in 67 mM sodium phosphate, 0.5% Tween 80, pH 7.4, as indicated. Subsequently, 9 μM Na_2S was added to perturb the steady state. Reaction monitored at 550 nm and 30°C. (B) Plot of α, the proportion of active enzyme in the inhibited state, against sulphide concentration. Conditions as in A, with 'α' calculated according to Eqn. 1; theoretical curves plotted for K_i = 1.0 μM (- - - - -) and 0.1 μM (· · · · · ·) according to a simple mass action law. (C) Rates of inhibition obtained from experiments as in A, plotted against sulphide concentration. $k(s^{-1})$ represents the first-order constant for the transition from the uninhibited to the partially inhibited steady state calculated in terms of α (Eqn. 1). Such a plot is of the 'Guggenheim' type, in which the slope has dimensions '$M^{-1} \cdot s^{-1}$' and reflects the apparent 'on' constant, whereas the intercept on the vertical axis, with dimensions 's^{-1}', reflects the apparent 'off' constant.

sium phosphate, pH 7.4, 0.25% Tween 80 buffer. Cytochrome *c* was type VI (horseheart) from Sigma Chemical Co.

The sulphide used was usually the sodium salt, $Na_2S \cdot 9\,H_2O$, Analar grade from BDH Chemicals Ltd. Occasionally, the ammonium salt was used, also obtained from BDH Chemicals (10% Analar $(NH_4)_2S$ solution). The sulphide concentration was determined in the stock solution sued each day by titration with a standard I_2 solution (BDH Chemicals) using soluble starch to determine the end-point.

Spectra were obtained with an Aminco DW-2 instrument, and kinetic studies were done using this instrument or a Gilford single-beam recording spectrophotometer. The cytochrome aa_3 concentration was calculated using ΔE_{mM} (605–630 nm), reduced minus oxidized, equal to 27 (equivalent to 13.5 mM^{-1} on a haem *a* basis).

Oxygen electrode studies were carried out with a Yellow Springs Instrument Co. Clark electrode attached to a suitable polarizing box and a Perkin-Elmer (Coleman) 165 recorder. The reaction vessel had a total volume of 4.2 ml.

Results

Fig. 1A illustrates the progressive inhibition of cytochrome *c* oxidase activity following addition of sulphide during the aerobic steady state monitored by cytochrome *c* reduction as described previously [3,11]. The time course and extent of inhibition at a given sulphide concentration can be determined using Eqn. 1 (aa_3 and *c* denote the corresponding cytochrome species):

$$\alpha = \frac{[aa_3]_t}{[aa_3]_0} = \frac{[c^{2+}]_0[c^{3+}]_t}{[c^{2+}]_t[c^{3+}]_0} \qquad (1)$$

Equilibrium ($t = \infty$) values for the amount of active enzyme present can be plotted as in Fig. 1B. Under the conditions employed inhibition by sulphide does not follow a simple binding function. At low sulphide levels, the estimated value of K_i is greater than 1.0 μM, while at higher sulphide livels, K_i approaches 0.1 μM. When the apparent rate constants for the transition to the inhibited state are plotted against sulphide concentration as in Fig. 1C, the resulting points fall upon a line, of which the intercept at low sulphide levels suggests a dissociation rate constant of approx. 0.01 s^{-1}. But the points at high sulphide levels suggest a much lower value. Association (on) and dissociation (off) constants of $1.2 \cdot 10^4\ M^{-1} \cdot s^{-1}$ and about $10^{-3}\ s^{-1}$, respectively, were reported previously [3] for sulphide under similar conditions. The maximum slope in Fig. 1C corresponds to an association rate constant of $7.5 \cdot 10^3\ M^{-1} \cdot s^{-1}$, but a meaningful value for the dissociation (off) rate constant cannot be obtained by this method.

Fig. 2A illustrates the results obtained using another method of studying the interaction between sulphide and enzyme. The spectroscopic changes which occur on adding sulphide to an aerobic solution of the resting enzyme are monitored in the Soret region. Fig. 2A shows the absorption increase at 438 nm relative to that at 417 nm occurring at various sulphide concentrations, and the apparent rate constants obtained are plotted against sulphide concentration in Fig. 2B. Below 3.0 μM, the rate constant is concentration independent, reflecting only the removal of the sulphide by a proportion of the enzyme present. Above 3.0 μM sulphide, the rate is proportional to sulphide concentration with a second-order rate constant of $1.4 \cdot 10^4\ M^{-1} \cdot s^{-1}$, essentially the same as that seen in the catalytic system (Fig. 1C).

However, spectroscopic and catalytic examination of the enzyme so treated reveals unexpected features. The spectrum of the sulphide-dependent change is not identical with that of the sulphide complex produced by excess of ligand [10]. As illustrated in Fig. 3A, it is characterized by a Soret maximum (difference spectrum) at 433 nm and a visible peak at 607 nm. This change, however, occurs with near stoichiometry (Fig. 3B), only a slight excess of sulphide being required to complete the spectroscopic change monitored at 433–417 nm. 1 mol of sulphide is apparently sufficient to transform 1 mol of cytochrome aa_3.

The resulting 'complex' can be tested both directly and after column chromatography (to remove excess sulphide) for catalytic activity. In a polarographic system where addition of excess sulphide produces a substantial inhibition, the sulphide-modified protein shows identical catalytic behaviour to that of untreated enzyme (Fig. 4). This cannot readily be attributed to rapid dissociation of sulphide, as both spectroscopic determinations [3] and the kinetic analysis

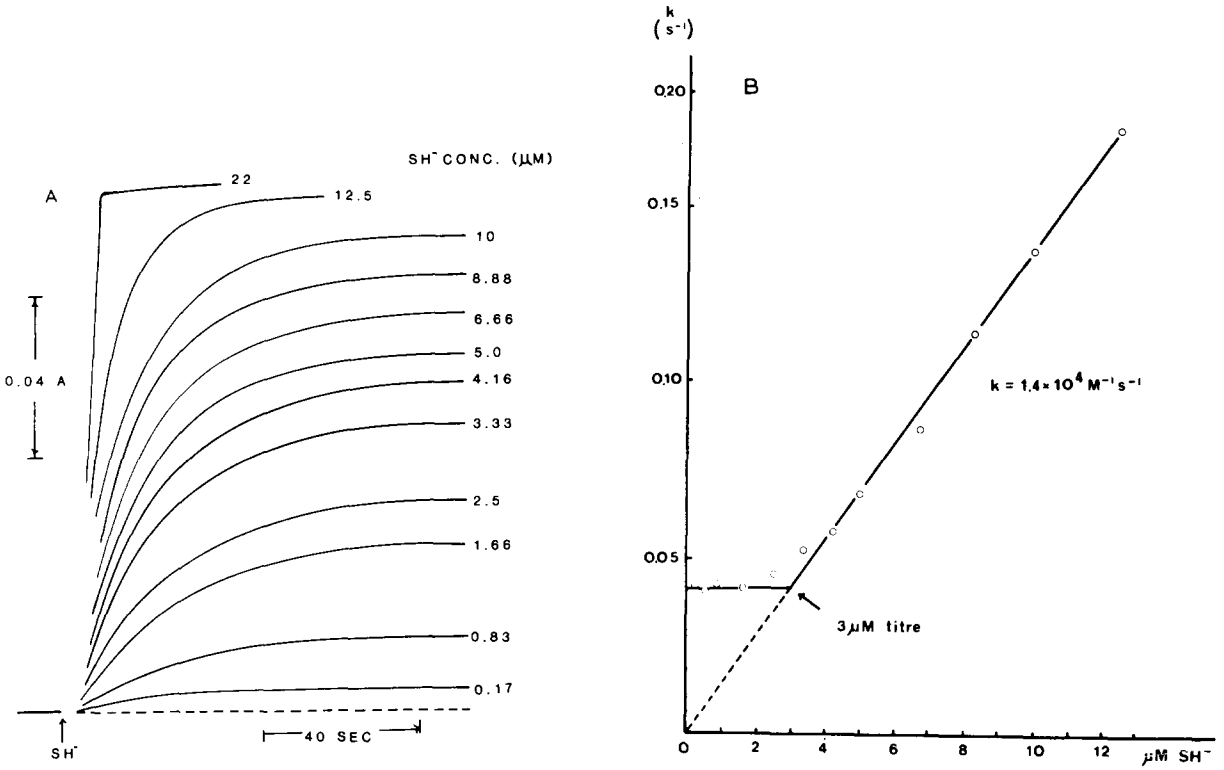


Fig. 2. Spectrophotometric effect of sulphide on cytochrome aa_3. (A) Absorption changes at 433–417 nm induced by the addition of sulphide to enzyme. The indicated concentrations of Na_2S were added to 3.5 μM cytochrome aa_3 in 67 mM sodium phosphate, pH 7.4, 0.25% Tween 80 at 30°C, and the reaction monitored aerobically by dual-wavelength spectroscopy. (B) Rate constants for the spectrophotometric reaction in A plotted against sulphide concentration. The $k(s^{-1})$ axis represents the apparent first-order constants for the transitions from the free to the fully or partially modified enzyme.

(Figs. 1C and 2B) indicate that this is slow. It thus seemed important to compare the spectra of the products obtained at low and high levels of sulphide, anaerobically as well as aerobically, as shown in Fig. 5.

Anaerobically, the addition of sulphide to 'resting' ferric enzyme gives rise to the appearance of a 'low-spin' ferric-type spectrum not identical with that seen on the addition of excess sulphide to the enzyme aerobically (compare the dotted curve with the dot-dashed curve in Fig. 5A). On addition of oxygen a spectrum is seen with the α-peak at 607 nm (dashed curve in Fig. 5A). A related sequence of changes can be followed under aerobic conditions, where, however, formation and decay of the 607 nm complex seem to occur concurrently; Fig. 5B shows the initial sulphide-induced spectrum (dotted line), together with the final product at low sulphide levels (continuous line). The latter can be converted into a further species by addition of excess sulphide (dashed and dot-dashed spectra); the final form seems to be the true inhibited form of the enzyme previously reported [3,10].

Fig. 6 illustrates the oxygen uptake that occurs simultaneously with the initial sulphide-enzyme interaction. Successive aliquots of Na_2S solution (Fig. 6A) induce uptake of approximately stoichiometric amounts of O_2; as the sulphide addition is continued the rate of O_2 uptake declines, while the amount taken up increases. Initially between 2 and 3 mol O_2 are taken up per mol of SH^- added; after an amount of SH^- has been added equimolar with the cytochrome aa_3 present (Fig. 6B), the slower O_2 uptake reflects approx. 1 mol O_2 per mol SH^-.

Discussion

The results described here appear to show that:

(a) the initial product of sulphide-cytochrome aa_3

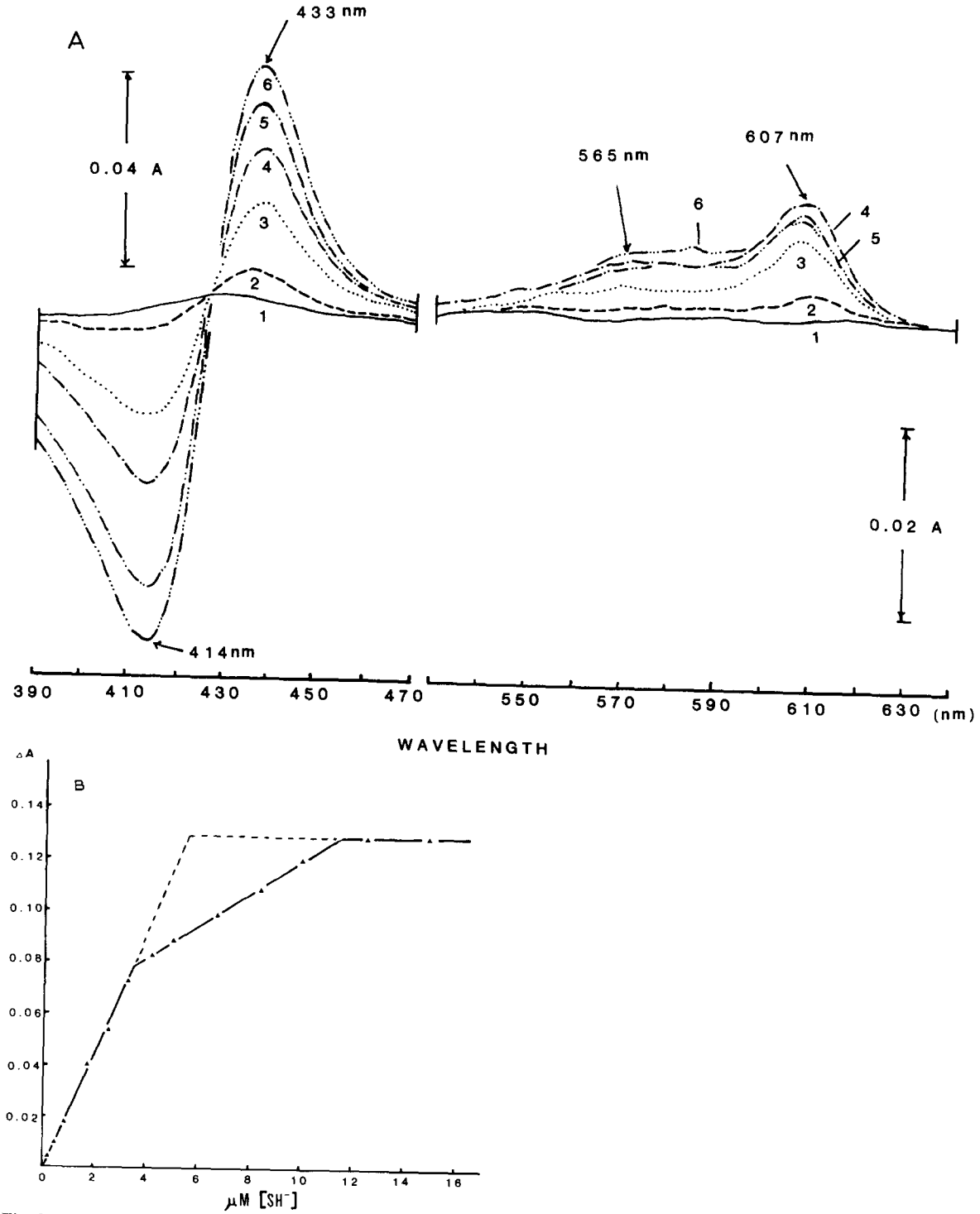


Fig. 3. Aerobic titration of cytochrome aa_3 by sulphide. (A) Difference spectra obtained upon addition of varying amounts of sulphide to an aerobic solution of enzyme. 3.5 μM cytochrome aa_3 in 67 mM sodium phosphate, 0.25% Tween 80, pH 7.4, was titrated with (1) 0, (2) 0.83 μM, (3) 3.3 μM, (4) 6.7 μM, (5) 10 μM and (6) 13 μM Na_2S, at 30°C. The reference cuvette contained oxidized (resting) enzyme. (B) Total absorbance change at 433–417 nm from experiments as in A plotted against sulphide concentration. All conditions as in A.

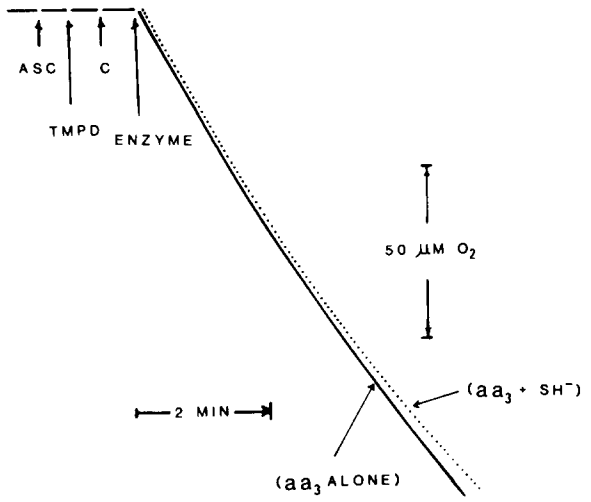


Fig. 4. Polarographic assay of oxygen uptake by native cytochrome aa_3 and its sulphide-modified from. 18.5 nm cytochrome aa_3 (aa_3) was added to 7 mM ascorbate (ASC); 178 μM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) and 4.8 μM cytochrome *c* (*c*) in 67 mM sodium phosphate, 0.25% Tween 80, pH 7.4, at 30°C. In one experiment (- - - - -) the enzyme (5 μM) was preincubated with 5 μM sulphide in 67 mM phosphate, 0.25% Tween 80, pH 7.4, at 30°C for 2 min, before addition to the oxygen electrode chamber.

interaction is not an inhibited form of the enzyme; and–

(b) more than 1 mol sulphide/mol cytochrome aa_3 is needed to secure inhibition; but–

(c) the rate of initial spectroscopic change is closely similar to the rate of inhibition; and furthermore–

(d) the initial change is both oxygen dependent and characterized by O_2 uptake, during which–

(e) a 607 nm form of the enzyme appears, which may be converted into a catalytically active low-spin (oxyferri) * state.

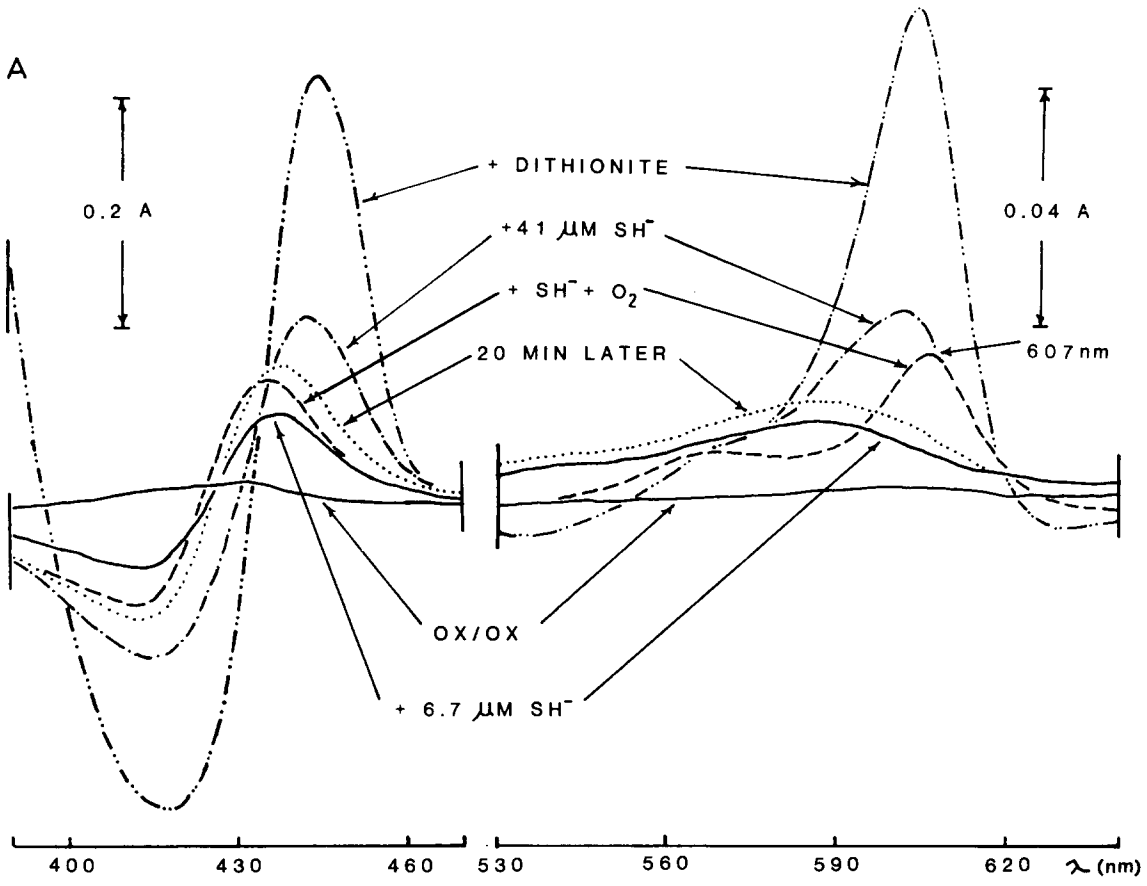
We interpret these findings as follows (see Fig. 7). The complex obtained upon adding sulphide to the resting enzyme is an essentially low-spin form which in the presence of oxygen can be transformed via the unstable '607 nm complex' to the 'oxyferri' form of the enzyme. Steady-state enzyme activity generates at least two other sulphide species, a stable fully oxidized form and the half-reduced form. We thus expected that sulphide addition to the resting enzyme under anaerobic conditions would give rise to an identifiably ferrous species; as Fig. 5 shows, this was not the case (cf. the results obtained by Nicholis and Chanady [14] using carbon monoxide as inhibitor/reductant). We conclude that the half-reduced form (bracketed) is not produced stoichiometrically, but is present in a quasi-equilibrium displaced by the rapid reaction of O_2 to give the 607 nm complex (compound C).

It is also clear that there must be at least two forms of sulphide-complexed ferric enzyme, the unstable form leading to ferrous enzyme, and a stable form responsible for inhibition even when cytochrome *a* is in the ferric state. If only the partially reduced form (cytochrome $a^{2+}a_3\ ^{3+}SH^-$) were inhibited, a continuous 'leak' through to oxygen would be expected when cytochromes *a* and *c* were partially reduced, which is not observed experimentally.

The conclusion that cytochrome *c* oxidase can utilize H_2S as a substrate as well as an inhibitor may throw some light on some anomalous behaviour seen with this reagent. Wever et al. [6] and Wilson et al. [5] both reported a 'split' EPR spectrum for the half-reduced enzyme at high sulphide levels (*g* 2.57–2.60, 2.26 and *g* 2.54, 2.22–2.23, 1.87). Shaw et al. [7], starting with reduced enzyme and partially reoxidizing it with porphyrexide, identified an early and unstable product of the sulphide reaction (*g* 2.77, 2.23, 1.7) preceding the final stable half-reduced form (*g* 2.54 species). Seiter and Angelos [8] identify part of the anomaly as a free radical signal. Fig. 7 indicates at least three species (cytochrome $a^{2+}a_3\ ^{3+}H_2S$, cytochrome $a^{3+}a_3\ ^{3+}H_2S$ (oxyferri, or low-spin), and cytochrome $a^{3+}a_3\ ^{3+}H_2S$) potentially capable of showing low-spin signals. In addition, electron transfer giving rise to sulphur radicals (cytochrome $a^{3+}a_3\ ^{2+}SH^{\cdot}$) may account for the extra signal seen by Seiter and Angelos [8].

Petersen's finding [9] that sulphide, like cyanide and unlike azide and formate, shows non-competitive inhibition kinetics with respect to oxygen concentration may also be explicable on such a scheme. Such

* The term 'oxyferri' is used [15] to denote the low-spin form of oxidized enzyme produced by oxygenation of partially reduced enzyme, to distinguish it from the 'oxyferro' complex produced at low temperatures [16]. The oxyferri form probably does not contain any part of the oxygen molecule involved in its formation.


A
0.2 A
0.04 A
+ DITHIONITE
+41 μM SH⁻
+ SH⁻ + O₂
20 MIN LATER
607nm
OX/OX
+ 6.7 μM SH⁻
400
430
460
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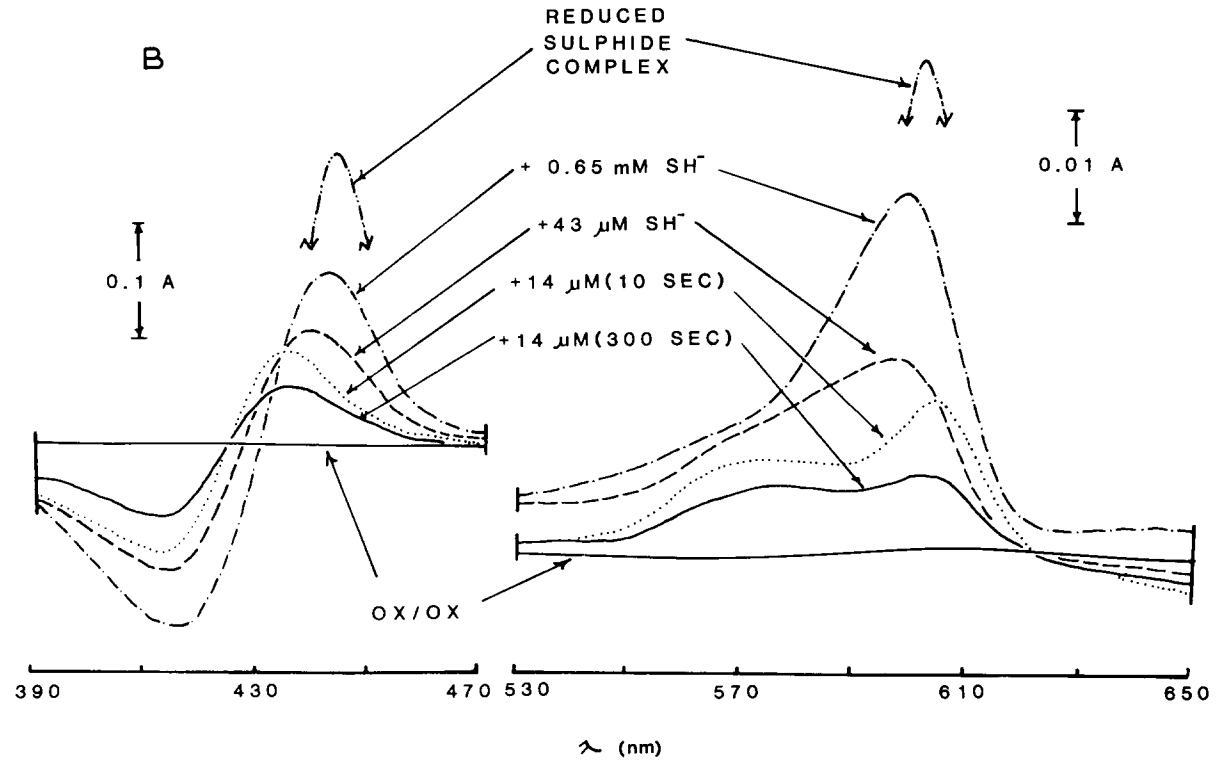
560
590
620
λ (nm)


B
REDUCED
SULPHIDE
COMPLEX
0.01 A
0.1 A
+ 0.65 mM SH⁻
+43 μM SH⁻
+14 μM(10 SEC)
+14 μM(300 SEC)
OX/OX
390
430
470
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610
650
λ (nm)

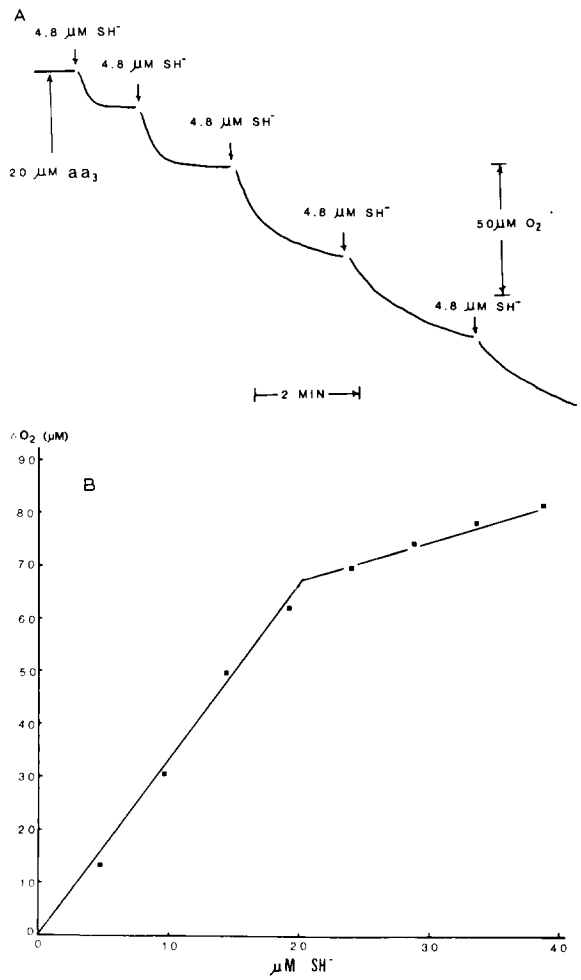

Fig. 6. Titration of oxygen with sulphide in the presence of cytochrome aa_3. (A) Polarographic tracing of O_2 uptake upon successive sulphide additions in the presence of enzyme. 20 μM cytochrome aa_3 (aa_3) in 67 mM sodium phosphate, 0.25% Tween 80, pH 7.4, was titrated with successive additions of 4.8 μM Na_2S at 30°C as indicated in an O_2 electrode chamber. (B) Plot of oxygen uptake against added sulphide concentration. Experimental conditions as in A. Note the break in the plot at approx. 20 μM sulphide.

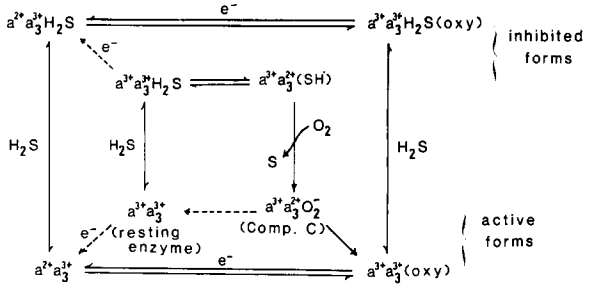

Fig. 7. A scheme for the interactions between sulphide and cytochrome aa_3. The inner cycle indicates the proposed 'oxidative' route for sulphide-cytochrome aa_3 interaction; the outer cycle indicates the reactions leading to enzyme inhibition. 'Comp. C' refers to the corresponding intermediate produced at low temperatures [16] or at room temperature [15] by treating partially reduced enzyme with oxygen. 'oxy' refers to the oxyferri form of the enzyme (see footnote, p. 317) to which the 607 nm species (Comp. C) decays; it may be capable of reacting directly with sulphide to give the inhibited form cytochrome $a^{3+}a_3{}^{3+}H_2S$(oxy). (See text for explanation of the other indicated reactions.)

kinetics require that the vulnerable form of the enzyme be a species other than the one depleted at low oxygen concentration. If a partially reduced or partially reoxidized form (cytochrome $a^{2+}a_3{}^{3+}$) rather than the resting oxidized form (cytochrome $a^{3+}a_3{}^{3+}$) is the sulphide-sensitive species (cf. Shaw et al. [7]), then apparent non-competitive kinetics could result.

The ready transfer of electrons from sulphide to haem iron in the resting enzyme, together with the existence of stable liganded ferric forms in the 'mixed valence' and probably in the oxyferri state (Fig. 7) raises some interesting questions concerning the configuration at the cytochrome a_3 haem iron. The inorganic SH^- may form a bridge between Fe_{a3} and Cu_{a3} in the resting state which facilitates two-electron reduction of the two metal centres. The corresponding complex obtained from the oxyferri state or the

Fig. 5. Spectra of sulphide-induced complexes of cytochrome aa_3 at low and high sulphide concentrations. (A) Complexes formed anaerobically. 3.7 μM cytochrome aa_3 in 67 mM sodium phosphate, 0.25% Tween 80, pH 7.4, at 30°C in a Thunberg cuvette under vacuum was treated with 6.7 μM Na_2S to give an immediate (———) and a 20-min (· · · · ·) spectrum. Admission of air produced the 607 nm form (- - - - -). Further treatment with 41 μM Na_2S gave the new spectrum (· – · – ·) and finally dithionite reduction the spectrum of reduced cytochrome *a* (· · – · · – · ·). (B) Complexes formed aerobically 4.6 μM cytochrome aa_3 in an aerobic cuvette was treated with 14 μM Na_2S in 0.1 M sodium phosphate, 0.25% Tween 80, pH 7.4, at 30°C to give an initial (10 s) spectrum (· · · · ·) and a spectrum after 5 min (———). 43 μM Na_2S gave rise to the spectrum (- - - - -) and 0.65 mM Na_2S to a product in which cytochrome *a* is partly reduced (· – · – ·). The positions of the dithionite-reduced sulphide complex peaks are also indicated (· · – · · – · ·).

mixed-valence state must be unable to accept the two electrons. Either the copper associated with cytochrome a_3 (Cu_{a3}) is reduced in such states, contrary to Nicholls and Holdebrandt [17] but in accordance with the suggestion of Hohnson et al. [18], or this Cu is no longer in close proximity to the bound sulphide. It may also be noted that an organic sulphide group from the protein has recently been proposed as bridging ligand vetween Fe and Cu in the cytochrome a_3 centre [19]. The reaction with inorganic sulphide may therefore also provide clues to the functioning of the enzyme in its uninhibited state.

Acknowledgements

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