Interactions of sulphide and other ligands with cytochrome *c* oxidase

An electron-paramagnetic-resonance study

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The effect of sulphide on resting oxidized cytochrome *c* oxidase was studied by both e.p.r. and optical-absorption spectroscopy. Excess sulphide causes some reduction of cytochrome *a*, Cu_A and Cu_B, and the formation of the cytochrome *a*₃-SH complex after about 1 min. After several hours in the presence of excess sulphide only the e.p.r. signals due to low-spin ferricytochrome *a*₃-SH persist, giving a partially reduced species. Re-oxidation of this partially reduced sulphide-bound enzyme by ferricyanide makes all of the metal centres except Cu_B detectable by e.p.r. We conclude that sulphide has reduced and binds to Cu_B as well as to ferricytochrome *a*₃. Sulphide binding to cuprous Cu_B may raise its mid-point potential and make re-oxidation difficult. Addition of reductant (ascorbate + *NNN'*-tetramethyl-*p*-phenylenediamine) and sulphide together to the oxidized resting enzyme produces a species in which cytochrome *a* and Cu_A are nearly completely reduced and cytochrome *a*₃ is e.p.r.-detectable as approx. 80% of one haem in the low-spin sulphide-bound complex. The *g* = 12 signal of this partially reduced derivative is almost unchanged in magnitude relative to that of the resting enzyme; this suggests that the *g* = 12 signal may arise from less than 20% of the enzyme and that it may be relatively unreactive to both ligation and reduction. Such a reactivity pattern of the *g* = 12 form of the oxidase is also demonstrated with the ligands F⁻ and NO, which are thought to bind to cytochrome *a*₃ and Cu_B respectively.

Sulphide was first used as a ligand for cytochrome *c* oxidase by Keilin (1929). It has been shown to be a reductant of the enzyme only comparatively recently (Wever *et al.*, 1975). The e.p.r. signal of low-spin ferricytochrome *a*₃, when complexed by azide, sulphide or cyanide, is only seen in the partially reduced state of the enzyme, presumably with Cu_B reduced (Van Gelder & Beinert, 1969; Wever *et al.*, 1975). Nicholls and co-workers (Nicholls, 1975; Nicholls & Kim, 1981,

1982) have demonstrated a number of spectrophotometric anomalies with the use of sulphide as a ligand to cytochrome oxidase that distinguish it from other ligands of the enzyme. Sulphide binding to cytochrome *a*₃ induces a blue-shift in the spectrum of reduced cytochrome *a*. The Soret spectrum of the partially reduced sulphide-bound enzyme is more intense at 445 nm relative to the analogous complexes with azide and cyanide (Nicholls, 1975). Furthermore, it has been suggested that sulphide is able to reduce the cytochrome *a*₃-Cu_B centre directly and that up to 3 mol of sulphide/mol of *aa*₃ is required for inhibition (Nicholls & Kim, 1981). We considered that it would be useful to follow up some of this visible-region spectroscopy with a more-detailed study by e.p.r. spectroscopy.

In the course of this work we have also made observations on the '*g* = 12' signal seen with the oxidized resting enzyme and on changes in this

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signal during the reaction of the enzyme with sulphide and other ligands and reductants. Hagen (1982) has suggested that this signal arises from cytochrome a_3 in the $S = 2$ spin state, possibly indicating haem in the high-spin ferryl oxidation state, and that this signal is to be quantified as 1 equivalent/mol of enzyme. Such an interpretation lends support to the idea advanced by Sieter & Angelos (1980) that Cu_B is cuprous in all redox states of the enzyme and that ferryl $\text{Fe } a_3$ is a two-electron acceptor. Much of the argument of Seiter & Angelos (1980) rested on experiments conducted with sulphide-bound cytochrome c oxidase.

In the present paper we report e.p.r. experiments that suggest that sulphide is able to reduce Cu_B directly and then after this is able to bind to both cuprous Cu_B and ferricytochrome a_3 . The binding of sulphide to reduced Cu_B apparently raises its mid-point potential so that it is not re-oxidized appreciably by ferricyanide. It is this type of result that led Seiter & Angelos (1980) to suggest that Cu_B does not undergo oxidation and reduction during the catalytic cycle of the unliganded enzyme. In addition, we demonstrate with sulphide, fluoride and NO that the ' $g = 12$ ' signal does not account for all the cytochrome a_3 , in contrast with the suggestion by Hagen (1982). The ' $g = 12$ ' form of our enzyme is sluggish in its responses to both ligation and reduction.

Materials and methods

Cytochrome c oxidase was prepared from bovine heart by the method of Yonetani (1960) as modified in this laboratory (Thomson *et al.*, 1981). An alteration to this procedure during its final stages produced an enzyme sample that in the resting oxidized state was reactive with NO. The alteration consisted of resuspending the enzyme in 1% Triton X-100/0.1 M-sodium phosphate buffer, pH 7.4, at a protein concentration of about 15 mg/ml, after it had been through one cycle of $(\text{NH}_4)_2\text{SO}_4$ fractionation in buffer containing 0.5% Tween 80. The enzyme solution in Triton X-100/phosphate buffer was frozen slowly at -20°C . The sample was re-thawed, adjusted to 22% saturation with $(\text{NH}_4)_2\text{SO}_4$ and centrifuged at 20000 *g* for 10 min. A colourless precipitate was obtained. The enzyme was then precipitated at about 26% saturation with $(\text{NH}_4)_2\text{SO}_4$ and resuspended in buffer containing 0.25% Tween 80.

Na_2S , NaF , KCN , $\text{Na}_2\text{S}_2\text{O}_4$, NO and $(\text{NH}_4)_2\text{SO}_4$ were obtained from BDH Chemicals, Poole, Dorset, U.K. Tween 80, Triton X-100, sodium ascorbate, NNN' -tetramethyl-*p*-phenylenediamine hydrochloride, Na_2HPO_4 and NaH_2PO_4 were from Sigma Chemical Co. (Poole, Dorset, U.K.).

E.p.r. spectra were recorded on either a Bruker ER-200D or a Varian E3 spectrometer. The Bruker instrument was fitted with an ESR-900 (Oxford Instruments) flow cryostat. Temperature regulation was achieved by a D.T.C.-2 temperature-controller (Oxford Instruments). The spectrometers were calibrated with reference to 1,1-diphenyl-2-picrylhydrazyl. Quantification of low-spin haem signals was done by the procedure of Aasa & Vänngård (1975) relative to a metmyoglobin cyanide standard. High-spin signals were quantified by the truncated double-integral method described by Aasa *et al.* (1976). Photolysis of e.p.r. samples was carried out *in situ* by focusing the white light from a slide projector, fitted with a 200 W lamp, on to the sample through a slotted wall of the cavity.

Absorbance spectra were measured with a Pye-Unicam SP.8-200 recording spectrophotometer.

Results

Sulphide reduction and ligation of cytochrome c oxidase

Fig. 1 shows the effect on the visible-absorption spectrum produced by adding excess sulphide to an aerobic sample of the enzyme. In difference spectra, relative to the oxidized resting enzyme, a band at 605 nm appears. This is accompanied by an increase in absorption in the Soret region at 445 nm (not shown). This behaviour is largely due to the reduction of cytochrome a (and probably Cu_A), which is almost 40% complete after 1 min. After 10 min at 20°C following the addition of sulphide the visible-absorption change is 80% complete relative to the level obtained after 1.5 h. The absorption coefficient at 605 nm ($\Delta\epsilon_{\text{red.-ox.}}^{605}$) of $21.5 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ is about 85% that found for the unliganded enzyme (Van Gelder & Beinert, 1969) and indicates that cytochrome a is nearly fully reduced. Under these conditions and on this time scale the spectral anomalies reported by Nicholls & Kim (1981) are not seen. Re-oxidation of this species by ferricyanide leaves a band centred at 590 nm that is presumably due to the ferricytochrome a_3 -SH species. The absorption coefficient for this complex at 590 nm ($\Delta\epsilon_{a_3\text{-SH-}a_3}^{590}$) is about $7.0 \text{ mm}^{-1} \cdot \text{cm}^{-1}$, not unlike values for other low-spin complexes of cytochrome a_3 .

Fig. 2 shows e.p.r. spectra of the oxidase recorded before (trace *a*) and at different times after the addition of sulphide (traces *b-e*) to an aerobic sample of the enzyme. Different regions of each spectrum are recorded at different gain values and these are noted in the Figure legend. In spectrum (*a*), which is characteristic of the oxidized enzyme, there are signals at $g = 3.0$, 2.2 and 1.5 due to ferricytochrome a and at $g = 2.0$

Fig. 1. Optical difference spectra after the addition of sulphide to oxidized resting cytochrome *c* oxidase. The oxidase concentration was $4.16 \mu\text{M}$ and the buffer was 100 mM-sodium phosphate, pH 7.4, containing 0.25% Tween-80. Na_2S was added to final concentration of 1.10 mM. The spectra in the Figure were measured with reference to a baseline with the resting oxidized enzyme in both cuvettes. Solid $\text{K}_3\text{Fe}(\text{CN})_6$ was added to re-oxidize the excess S^{2-} ion.

from oxidized Cu_A . There are also small signals near $g = 6.0$ and $g = 4.3$ assigned to oxidized high-spin cytochrome a_3 and to contaminating iron respectively. But a feature of the oxidase spectrum that has recently gained attention is found at very low field with an apparent g -value of 12. This signal could arise either from the coupled centre formed by cytochrome a_3 and Cu_B (Greenaway *et al.*, 1977; Brudvig *et al.*, 1981) or from cytochrome a_3 alone in the high-spin ferryl oxidation state (Hagen, 1982).

Trace (b) in Fig. 2 is the e.p.r. spectrum of the oxidase recorded after a 1 min incubation at 20°C with sulphide. The signals at $g = 3.0$ and $g = 2.0$ have both diminished in intensity at this time, indicating the reduction of cytochrome *a* and Cu_A , in accordance with the optical spectrum in Fig. 1. However, at the higher enzyme concentration required for e.p.r. the rate of the reaction of the enzyme is lower than under the conditions of the spectrophotometric experiments. A new signal due to low-spin haem has appeared in this spectrum (b), with g -values of 2.53, 2.23 and 1.86, due to the formation of some cytochrome a_3 -SH, presumably in parallel with the reduction of Cu_B . It also appears that the unusual signal at ' $g = 12$ ' has increased in size at this time. We are cautious of overemphasizing this result, since the intensity of this signal is known to be very temperature-

sensitive in the range 4–15K (Brudvig *et al.*, 1981). Spectrum (b) was recorded under the same, nominal, conditions as those for spectrum (a), but the experiment did require the sample to be removed from the cryostat, thawed and then re-frozen. These manipulations could result in different temperature and/or power in the sample cavity. However, with these caveats in mind, it is possible that this effect on the ' $g = 12$ ' signal is caused by an interaction of sulphide with the cytochrome a_3 - Cu_B centre related to the optical changes reported by Nicholls & Kim (1981, 1982).

After 1 h (Fig. 2, trace c) the reduction of cytochrome *a* and Cu_A has increased. The signal due to cytochrome *a* is only 30% of that seen in the oxidized resting enzyme, although there is 60% of oxidized Cu_A remaining. The intensity of the ferricytochrome a_3 -SH-complex signal is greater than 50% of that from a partially reduced sulphide-bound sample when fully formed (see Fig. 3). But the signal at ' $g = 12$ ' is still about the same magnitude as in the resting oxidized enzyme.

Trace (d) in Fig. 2 was recorded after the same sample had been warmed at 20°C for 3 h. Relative to trace (c) both cytochrome *a* and Cu_A are more oxidized whereas the signals due to ferricytochrome a_3 -SH and the ' $g = 12$ ' component have diminished. There has been a large increase in a signal near $g = 6.0$, due to high-spin ferric haem. It

Fig. 2. E.p.r. spectra of cytochrome *c* oxidase recorded after the addition of sulphide to an aerobic sample of resting oxidized enzyme

The enzyme concentration was $516 \mu\text{M}$ and Na_2S was added to a concentration of 3.5 mM in spectra (b) through to (d). The buffer was the same as that specified in the legend to Fig. 1. Microwave frequency was 9.43 GHz and power 2.01 mW . The modulation amplitude was 0.63 mT at a frequency of 100 kHz . Each spectrum was recorded in 200 s with a time constant of 0.5 s . The temperature for all spectra was 10 K . The gain setting for each scan was as follows: (a) 8×10^5 from 0 to 0.2 T , 2×10^5 from 0.2 to 0.27 T and 3.2×10^4 from 0.27 to 0.53 T . (b) Same as in (a) except that from 0.2 to 0.27 T the gain was 2.5×10^5 . (c) same as (b). (d) Same as (b) except that the gain was 2.5×10^5 from 0.35 to 0.53 T . (e) Same as (d).

resembles closely in form the high-spin signal seen in the partially reduced liganded enzyme (Aasa *et al.*, 1976), with g -values of 6.34, 5.86 and 5.50. It probably arises from liganded oxidized cytochrome a_3 centres in which the adjacent Cu_B has remained reduced. Aasa *et al.* (1976) had suggested from simulation studies that such a form was a composite of signals from three slightly different high-spin centres. The intensity of our high-spin signal is about 7.5% of one haem; that it arises from cytochrome a_3 is demonstrated in trace (e) in Fig. 2. When the mixture (trace d) was supplemented with fresh sulphide and immediately frozen (i.e. after about 30 s), the high-spin signal had vanished, whereas the signal due to ferricytochrome a_3 -SH increased (trace e). The signals due to Cu_A and cytochrome a both decreased upon the second sulphide addition. A substantial signal at $g = 12$ is still present.

Re-oxidation of partially reduced sulphide-inhibited cytochrome c oxidase

Fig. 3(a) shows the e.p.r. spectrum of cytochrome c oxidase incubated with an excess of sulphide for 12 h at 4°C (continuous trace). The major signals are those due to the ferricytochrome a_3 -SH complex, with g -values of 2.53, 2.23 and 1.86. There is also present a slightly more rhombic low-spin haem signal with its low-field g value at 2.59. This spectrum is similar to that reported by Wever *et al.* (1975). It is featureless in the ' $g = 12$ ' region (not shown), and the major low-spin signal accounts for 80% of one haem in the complex. When excess ferricyanide is added to this sample, additional signals appear (dashed trace in Fig. 3a). There are two low-spin haem signals at $g = 3.0$ and $g = 2.53$, attributable to cytochrome a and to sulphide-bound cytochrome a_3 . These signals are obscured by a very broad signal centred at $g = 2.68$, which is due to the excess of ferricyanide present. The signal of oxidized Cu_A at $g = 2.0$ has also re-appeared. The persistence of cytochrome a_3 in an e.p.r.-detectable form in this experiment (Fig. 3a) contrasts with its behaviour during an analogous re-oxidation experiment with the partially reduced cyanide-bound enzyme (Fig. 3b). In the latter Figure the $g = 3.0$ region of the oxidase e.p.r. spectrum is shown for oxidized (continuous trace) and partially reduced (dashed trace) forms of the cyanide-bound enzyme. When the oxidized cyanide-bound enzyme is reduced with dithionite the signals at $g = 3.02$ and $g = 2.0$ (not shown) disappear, whereas a new signal at $g = 3.58$ due to the ferricytochrome a_3 -CN complex appears (cf. Johnson *et al.*, 1981). When a stoichiometric amount of ferricyanide is added to this partially reduced sample, the cytochrome a_3 signal dis-

appears and that due to cytochrome a re-appears (dotted trace). In the case of the partially reduced sulphide-bound sample, an excess of the much stronger oxidant porphyraxide is necessary to cause a substantial decrease in the signal due to ferricytochrome a_3 -SH (dotted trace in Fig. 3a). The large signal at $g = 2.0$ seen in this spectrum is due to excess porphyraxide.

It may be worth noting that the signal due to ferricyanide seen in Fig. 3(a) is greatly intensified by the presence of the enzyme. At the same concentration and under the same conditions of temperature, power and gain the spectrum of ferricyanide alone is virtually featureless.

Comparison of the effects of sulphide, fluoride and NO on the ' $g = 12$ ' signal

Fig. 4 compares e.p.r. spectra of the partially reduced sulphide-bound oxidase obtained in two different ways. In spectrum (a) the reductants ascorbate + NNN' -tetramethyl- p -phenylenediamine were added to an aerobic sample of the oxidase at the same time as sulphide. The mixture was frozen in liquid N₂ after 2 min at 20°C. There is nearly complete reduction of both cytochrome a and Cu_A, and a large signal due to ferricytochrome a_3 -SH. The signal at ' $g = 12$ ' is about the same size as with the resting oxidized enzyme, shown in spectrum (a) Fig. 2. Trace (b) in Fig. 4 shows that only the signal due to the low-spin sulphide complex of cytochrome a_3 is seen after 4 h incubation at 20°C with a large excess of sulphide. The signal at $g = 12$ is completely gone. The $g = 2.53$ signal in spectrum (a) is about the same size as that in spectrum (b) (note the different gain settings) and represents 80% of one haem in the complex. Therefore we conclude that, since the $g = 12$ signal is relatively unaffected, it cannot represent more than 20% of the cytochrome a_3 in our preparation. Moreover, it is evidently slow to react with the reductants ascorbate + NNN' -tetramethyl- p -phenylenediamine and the ligand sulphide.

A similar pattern of reactivity is seen with the ligands F⁻ and NO. Experiments analogous to those described above were conducted with the high-spin ligand F⁻. When NaF is added to the oxidized resting enzyme a small high-spin signal appears at $g = 6$ (< 5% of one haem in the complex) with little effect on any of the other signals. Upon the addition of reductants both the signals due to cytochrome a and to Cu_A largely disappear. The signal at $g = 6$ does not change in intensity significantly, but becomes slightly more rhombic in form. The ' $g = 12$ ' signal has remained unchanged during this treatment.

The resistance of the species with the ' $g = 12$ ' signal to reaction with ligands is perhaps most

Fig. 3. E.p.r. spectra during the formation and re-oxidation of partially reduced complexes of cytochrome *c* oxidase (a) E.p.r. spectra of sulphide complexes of cytochrome oxidase. The enzyme concentration was $190\text{ }\mu\text{M}$ in 100 mM -sodium/potassium phosphate buffer containing 0.25% Tween 80 in $^2\text{H}_2\text{O}$ mixed with an equal volume of deuterated ethanediol. —, Partially reduced sulphide complex obtained by incubation of the oxidized enzyme anaerobically with 4 mM - Na_2S for 24 h at 4°C ; ----, partially reduced sample re-oxidized with 5 mM - $\text{K}_3\text{Fe}(\text{CN})_6$; ·····, partially reduced sample re-oxidized with a few grains of solid porphyraxide. Microwave frequency was 9.43 GHz , power 2.0 mW , and modulation amplitude 0.63 mT . The scan time was 200 s with 1 s time constant, and the temperature was 10 K . The gain was 4×10^5 for the partially reduced sample and 1.25×10^5 for the re-oxidized samples. (b) Re-oxidation of the partially reduced cyanide complex of cytochrome oxidase. The enzyme sample ($175\text{ }\mu\text{M}$ in a_3) was incubated for 40 h at 4°C with 10 mM - KCN . The spectra are for oxidized cyanide-bound enzyme (—), after reduction with $750\text{ }\mu\text{M}$ - $\text{Na}_2\text{S}_2\text{O}_4$ (----) and after re-oxidation with 2.3 mM - $\text{K}_3\text{Fe}(\text{CN})_6$ (·····). Microwave frequency was 9.43 GHz , power 2.0 mW and modulation amplitude 0.63 mT . The temperature was 13 K . The gain was 6.2×10^5 .

easily seen in an experiment with NO, as shown in Fig. 5. The addition of NO to oxidized resting enzyme induces appearance in some forms of the enzyme of a high-spin haem signal whose magnitude depends on the preparative method employed (Brudvig *et al.*, 1981). This signal is thought to arise

as a consequence of NO binding to Cu_B , which quenches the magnetic interaction between Cu_B and cytochrome a_3 . Boelens *et al.* (1982) have reported that this complex is photosensitive. A series of analogous photolysis experiments is shown in Fig. 5. NO induces a high-spin signal in

Fig. 4. *E.p.r. spectra of the partially reduced sulphide-bound oxidase produced with both reductant and ligand and by incubation in the presence of sulphide alone*

The sample conditions are the same as those given in the legend to Fig. 2. Ascorbate (7.50 mM), *NNN'*-tetramethyl-*p*-phenylenediamine (TMPD) (250 μ M) and Na₂S (8.75 mM) were added simultaneously in (a) to an aerobic sample of the enzyme, which was frozen in liquid N₂ after 1 min at 20°C. The spectrometer settings are the same as those given in the legend to Fig. 2. In (b) the enzyme was incubated with 15 mM-Na₂S for 4 h at 20°C. In (a) the gain was 8.0×10^5 from 0 to 0.28 T and 1.25×10^5 from 0.28 to 0.53 T. In (b) the gain was 8×10^5 from 0 to 0.25 T, 2.5×10^5 from 0.25 to 0.285 T, 1.25×10^5 from 0.285 to 0.32 T, 3.2×10^4 from 0.32 to 0.355 and 1.25×10^5 from 0.355 to 0.53 T.

the oxidized resting enzyme, the extent of which is equivalent to 15% of one haem in the complex. This high-spin signal is abolished when the sample is exposed to white light for 10 min at 10 K, and it re-appears when the photolysed sample is held in the dark for 10 min at 50 K. During this cycle the intensity of the signal at ' $g = 12$ ' remained unchanged. This experiment demonstrates clearly that the species responsible for the ' $g = 12$ ' signal is quite unreactive towards NO. There is no ambiguity arising either from the difficulty of quantifying a signal of uncertain spin or from the extreme temperature-dependence of the intensity. The use of a light-beam as a probe provides the minimal interference with the experimental conditions under which the spectra are run.

Discussion

Sulphide reduction and binding of cytochrome c oxidase

It has been known for some time that sulphide is capable of acting as both a reductant and a ligand for cytochrome c oxidase. E.p.r. data presented in this paper suggest that sulphide may initially be a reductant of Cu_B and then ligate both cytochrome a_3^{3+} and Cu_B⁺. When sulphide is added to an aerobic sample of oxidase, cytochrome a , Cu_A and Cu_B are all reduced early in the reaction. This indicates that sulphide may be able to reduce Cu_B directly, in accordance with the suggestion by Nicholls & Kim (1981). When oxidase and sulphide mixtures are maintained aerobic the

Fig. 5. Effect of NO binding to Cu_B^{2+} and photolysis on the ' $g = 12$ ' e.p.r. signal of cytochrome *c* oxidase. The enzyme concentration was $166.3 \mu\text{M}$ of the 'Triton X-100' (see the text) in 100 mM-sodium phosphate buffer, pH 7.4, containing 0.25% Tween 80. —, Oxidized enzyme placed under an atmosphere of NO and immediately frozen; ----, exposure of the sample to white light for 5 min at 10 K; ·····, sample after being held in dark for 5 min at 50 K and subsequently frozen to 10 K. Microwave frequency was 9.42 GHz, power 2.0 mW and modulation amplitude 0.63 mT. The gain was 8×10^5 .

enzyme eventually oxidizes all the excess sulphide. After the exhaustion of sulphide, ferric cytochrome *a* and cupric Cu_A re-appear and cytochrome a_3 changes from a low-spin to a high-spin state but remains e.p.r.-detectable, indicating that some Cu_B is still reduced. The re-oxidation experiments of Fig. 3 demonstrate conclusively that sulphide treatment has altered Cu_B . The apparent redox potential of Cu_B after the reaction with sulphide must be greater than approx. 500 mV for it to resist oxidation by a large excess of ferricyanide. Analogous experiments with the cyanide-bound enzyme demonstrate that this behaviour is peculiar to the sulphide-treated oxidase. It seems unlikely that two CN^- ions bind to the cytochrome a_3^{3+} - Cu_B^{2+} site. This evidence leads us to suggest the reactions of the cytochrome a_3 - Cu_B centre with sulphide to be in accordance with Scheme 1.

The following points concerning Scheme 1 should be noted. (i) Reduction of cytochrome *a* and Cu_A^{2+} implicit in the catalytic cycle probably occurs via the classical reductive substrate site shared with ferrocytochrome *c*. (ii) The mechanism for inhibition (reactions 1–3) requires a stoichiometry of 3 SH^- per $a a_3$ molecule to produce the final inhibited state. This is in agreement with evidence on the stoichiometry of the reaction provided by Nicholls

& Kim (1981). (iii) Presumably the final form of partially reduced inhibited enzyme is unreactive towards O_2 , but the reactivity of the intermediate form with only cuprous Cu_B bound by sulphide is not known.

It is probable that only a fraction of the enzyme population reacts with sulphide in this way. This suggestion is borne out by the experiments on the ' $g = 12$ ' signal (see below). The ability of the enzyme solution to turn over excess sulphide implies that some a_3 - Cu_B remains O_2 -reactive. The ' $g = 12$ ' form, which has not bound sulphide, may be able to turn over excess sulphide by the conventional route involving cytochrome *a* reduction.

The inability of ferricyanide to re-oxidize Cu_B after sulphide treatment as reported in the present paper confirms the results reported by Seiter & Angelos (1980). These workers concluded, as a result of their observations, that Cu_B remains cuprous throughout the oxidase catalytic cycle even in the absence of added S^{2-} ion. However, the results given in the present paper show that the presence of the exogenous ligand is crucial. For example, re-oxidation of the partially reduced cyanide derivative of cytochrome *c* oxidase leads to the re-oxidation of Cu_B . Thus the conclusions of

Scheme 1

Seiter & Angelos (1980) cannot be generalized to all forms of cytochrome c oxidase. We suggest that the apparent mid-point potential of Cu_B is raised by the binding of sulphide to the cuprous form and that this prevents the re-oxidation of Cu_B .

The ' $g = 12$ ' and high-spin signals

Brudvig *et al.* (1981) provide spectroscopic evidence that isolated cytochrome c oxidase is heterogeneous. They suggest that there are three possible conformational states in any preparation of isolated enzyme. Only one of these states is characterized by the presence of a ' $g = 12$ ' signal. Evidence provided in the present paper involving sulphide binding, steady-state reduction in the presence of low-spin and high-spin ligands of cytochrome a_3 and NO binding to the oxidized enzyme, all supports the idea that the ' $g = 12$ ' signal arises from only a fraction of the enzyme population. During steady-state reduction of the enzyme by ascorbate + NNN' -tetramethyl- p -phenylenediamine in the presence of sulphide about 80% of cytochrome a_3 is e.p.r.-detectable and the ' $g = 12$ ' signal is very little changed from that seen with resting enzyme (see Figs. 2 and 4). In this respect we are in agreement with the conclusions of Brudvig *et al.* (1981). However, we have little to add concerning the origin of this signal,

except to note that the ' $g = 12$ ' form of the enzyme is slow to react with reductants and ligands relative either to the bulk of the resting enzyme or to cytochrome a_3 when in its e.p.r.-detectable ferric high-spin form.

The binding of F^- to cytochrome a_3 produces only a small amount of e.p.r.-detectable haem either in the oxidized or in the partially reduced forms of the enzyme. In the case of the oxidized enzyme this implies that F^- ligation to cytochrome a_3 is not strong enough to break the interaction assumed to be present between cytochrome a_3 and Cu_B in a large fraction of the enzyme. These experiments also imply that Cu_B remains oxidized during the fluoride-inhibited steady state. Similar behaviour is observed in the uninhibited steady state, where cytochrome a_3 remains largely e.p.r.-silent (Wilson *et al.*, 1982). This contrasts with that seen with the low-spin ligand azide, where cytochrome a_3 becomes e.p.r.-detectable during steady-state reduction, indicating that Cu_B is reduced (Van Gelder & Beinert, 1969).

Addition of NO to resting oxidized oxidase induces a high-spin haem signal from only a fraction of the oxidase. The amount of NO-reactive material is variable in different preparations of the isolated enzyme (Brudvig *et al.*, 1981). Thus in our standard Yonetani-type preparation

NO does not induce a high-spin signal when added to the resting oxidized form. The NO-reactive enzyme sample shown in Fig. 5 was produced by using a procedure similar to that employed by Hartzell & Beinert (1974), which was also the method used by Brudvig *et al.* (1981) to produce a large fraction of NO-reactive material.

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