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Cyanide inhibition of cytochrome *c* oxidase

A rapid-freeze e.p.r. investigation

Peter JENSEN, Michael T. WILSON,* Roland AASA and Bo G. MALMSTRÖM
Department of Biochemistry and Biophysics, University of Gothenburg and Chalmers University of Technology, S-412 96 Göteborg, Sweden

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The inhibition of cytochrome *c* oxidase by cyanide, starting either with the resting or the pulsed enzyme, was studied by rapid-freeze quenching followed by quantitative e.p.r. It is found that a partial reduction of cytochrome oxidase by transfer of 2 electron equivalents from ferrocytochrome *c* to cytochrome *a* and Cu_A will induce a transition from a closed to an open enzyme conformation, rendering the cytochrome a_3–Cu_B site accessible for cyanide binding, possibly as a bridging ligand. A heterogeneity in the enzyme is observed in that an e.p.r. signal from the cytochrome a_3^{3+}–HCN complex is only found in 20% of the molecules, whereas the remaining cyanide-bound a_3–Cu_B sites are e.p.r.-silent.

The inhibition of cytochrome *c* oxidase (EC 1.9.3.1) by cyanide remains an interesting problem half a century after its discovery. The present phase of study of this phenomenon was initiated by Van Buuren (1972), who in his comprehensive thesis summarized the earlier findings and also re-investigated the action of cyanide on the oxidase in order to establish the site of inhibition and its redox state. Among the four redox centres in the enzyme (cytochrome *a* and a_3, Cu_A and Cu_B), cytochrome a_3 has long been considered the binding site for cyanide as well as for many other inhibitors. A dilemma, however, has been the observation that the rate of cyanide binding to either the fully oxidized or the fully reduced enzyme is too slow to account for the rate of onset of inhibition in turnover experiments or with systems *in vivo*; recent work (Jones *et al.*, 1984) has established that the rates differ by as much as a factor larger than 10^5. From his experiments, Van Buuren (1972) concluded that the species susceptible to rapid inhibition is the partially reduced oxidase. He also suggested that partial reduction causes a conformational change that makes oxidized cytochrome a_3 more available to cyanide.

In stopped-flow experiments with reduced cytochrome *c* and O_2 (Antonini *et al.*, 1977) it was found that cytochrome oxidase can appear in two forms, which are optically similar but kinetically different: the 'resting' and the (kinetically faster) 'pulsed' form. The term 'pulsed' refers to the enzyme that has been freshly oxidized by the reaction of the reduced enzyme with O_2. In the absence of reducing substrates it slowly decays back to the resting form (Brunori *et al.*, 1981). The pulsed oxidase is related to another highly active enzyme form found by Sekuzu *et al.* (1959) and named 'oxygenated' oxidase by them. These findings opened the possibility that the enzyme forms with high activity are also the conformational states that bind cyanide rapidly, as had in fact been suggested by Brittain & Greenwood (1976) for the 'oxygenated' species.

This question has recently been addressed in detail in a re-investigation by Jones *et al.* (1984) of the kinetics of cyanide binding. They showed that a model with only two conformational states, resting and pulsed, is inadequate. An extended model was suggested, in which the enzyme in the pulsed as well as in the resting state can exist in a 'closed' or an 'open' conformation, of which only the latter reacts rapidly with cyanide. It was further proposed that the transition from the closed to the open conformation is triggered by the reduction of cytochrome *a* and Cu_A (and perhaps also Cu_B), and that the conformational change is followed by a rapid binding of cyanide to oxidized cytochrome a_3.

Although the model proposed by Jones *et al.*

* Visiting scientist of the Nobel Institute for Chemistry, 1983. Permanent address: Department of Chemistry, University of Essex, Wivenhoe Park, Colchester, Essex CO4 3SQ, U.K.

(1984) can adequately account for their extensive kinetic results, it would seem desirable to have more direct physical evidence to test the validity of its details. To this end we have followed the reaction of reduced cytochrome *c* with resting and with pulsed oxidase in the absence and in the presence of cyanide by e.p.r. spectroscopy with the aid of a rapid-freeze quenching technique. Our data show that the majority of the enzyme molecules in the resting oxidase react rapidly with cyanide when cytochrome *a* and Cu_A have been reduced, and this prevents entry of more than 2 electron equivalents from reduced cytochrome *c*. Also, with the pulsed oxidase only 2 electron equivalents can enter the enzyme in the presence of cyanide. In agreement with observations by Brudvig *et al.* (1981), we find, however, that the resting oxidase is heterogeneous. About 20% of the molecules are in a form in which the cyanide-inhibited enzyme has accepted 3 electron equivalents, as evidenced from the appearance of a low-spin Fe^{3+} e.p.r. signal from cytochrome a_3–HCN, showing that Cu_B must be reduced; this fraction of the molecules gives a high-spin Fe^{3+} signal in the absence of cyanide. Our results also indicate that the pulsed oxidase is heterogeneous, some molecules reacting rapidly with cyanide before both cytochrome *a* and Cu_A have been re-oxidized.

Materials and methods

Enzyme and chemicals

Cytochrome *c* oxidase was prepared from ox heart by the method of Van Buuren (1972). The enzyme contained 10nmol of haem A and 0.1–0.15mg of phospholipids (measured as lipid-bound P)/mg of protein. Absorbance ratios (typical) were: $A_{445(red.)}/A_{420(red.)} > 2.3$, $A_{605(red.)}/A_{554(red.)} > 2.3$, $A_{445(red.)}/A_{605(red.)} > 5.0$. The molecular activity was $20\,s^{-1}$, measured essentially as described by Smith & Conrad (1956). When the purified enzyme was incorporated into asolectin vesicles the activity rose to around $400\,s^{-1}$ (pH6.2, high ionic strength).

Enzyme concentrations were determined as described in Jensen *et al.* (1981). Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis by the procedure of Downer *et al.* (1976) gave a subunit composition similar to that described by Wikström *et al.* (1981) (seven major subunit bands).

Horse heart cytochrome *c* (type III) from Sigma Chemical Co. was used without further purification. Stock solutions of 2mM and 2.5mM reduced cytochrome *c* were prepared by titration with sodium ascorbate until the absorption band of cytochrome *c* at 695nm was completely bleached. The solution was then equilibrated with air or pure O_2. Inspection of the 695nm band showed no signs of re-oxidation. The amount of polymerized cytochrome *c* was less than 2%, determined from the difference in absorbance between ascorbate and dithionite-reduced cytochrome *c* at 550nm. Transferrin was kindly given by Dr. P. Aisen.

All experiments were performed in 50mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/NaOH buffer, pH7.4, at room temperature. Stock solutions of 0.48M-KCN were titrated with HCl to neutrality before use.

All chemicals were of analytical grade. All solutions were prepared fresh on the day of the experiment.

Spectroscopy

Optical spectra were recorded in a Beckman Acta MIV spectrophotometer. E.p.r. spectra at 15K and 9GHz were recorded with a Varian E-9 spectrometer equipped as described in Wilson *et al.* (1982). Four to eight spectra were collected from each sample and time-averaged to improve the signal-to-noise ratio before integration.

Rapid-freeze experiments

The rapid-freeze equipment and freezing procedures were identical with the description given in Wilson *et al.* (1982) except that the optical cell was used only as a container for the enzyme solution after mixing when delays were used. The T-mixer was also replaced by a tangential rapid-flow mixer, similar to the one described by Ballou & Palmer (1974). Gas-tight 2.5ml Hamilton syringes were used throughout the experiments (equal mixing).

The experiments starting with the resting enzyme were performed as follows. Syringe A was filled with 246μM-cytochrome oxidase solution in buffer equilibrated with air, and syringe B was filled with buffer only. An oxidized reference sample was then collected by mixing the contents of the two syringes in equal proportions. The buffer in syringe B was then replaced with 2mM aerated reduced cytochrome *c*. After collection of one sample without delay the cell compartment was attached and samples with different delay times were collected by using the multiple-push facility of the system. KCN was then injected in syringe B from the concentrated stock solution to give a final concentration of 20mM. The same number of samples were collected with the same delays as above.

Experiments with the pulsed enzyme were performed by reducing the cytochrome oxidase in syringe A (new enzyme preparation; 345μM) with an equivalent amount of sodium ascorbate and with a catalytic amount of cytochrome *c* present. After a reference sample had been collected by mixing cytochrome oxidase with the aerated buffer, syringe B was filled with O_2-bubbled

2.5mM reduced cytochrome *c* solution, and samples were taken as above. The cyanide addition was also made as with the resting enzyme. As internal standard 100 μM-transferrin (see below) was added to the cytochrome oxidase solution in syringe A before the pulsed experiments.

A second type of experiment with the pulsed enzyme was carried out by just mixing the reduced cytochrome oxidase with aerated cyanide-containing buffer with the same concentrations and delays as above.

Quantitative measurement of the rapid-freeze samples

It is for many reasons inevitable that difficulties arise when making quantitative e.p.r. measurements of rapid-freeze samples. The main problems occur during the packing of the powdered samples, where the sample concentration after packing can vary. This difference is due to variations in the packing force applied on the sample, and its packing characteristics. From studies of $CuSO_4$/EDTA solutions Ballou (1971) found a variation in packing factor of 6% with the same person packing the samples (packing factor defined as the ratio between the signal from a slowly frozen sample and a rapid-freeze one of the same material). It should be noted that this study was carried out with aqueous solutions of small ions, which pack easily. Uniform protein solutions poses no problems in packing, but, if chemical reactions that change the shape of the protein can take place, it can have large effects on packing ability. During the course of the experiment it was noted that samples captured during turnover were more difficult to pack compared with the resting oxidized cytochrome oxidase. Thus introducing delays before the reaction mixture was freeze-quenched led to such packing difficulties that we were forced to decrease the jet speed by 30% to be able to pack the samples at all. An interesting observation was made in that these difficulties were less pronounced after cyanide addition, possibly indicating an altered protein conformation after cyanide binding to the turnover enzyme.

Isopentane adhering to the outside of the e.p.r. tubes after freeze-quenching also causes variations, as it can be difficult to wipe off without heating the sample by the resultant friction. Remaining isopentane can lead to increased frost attraction on the tubes from humid air, thus lowering the sensitivity of the e.p.r. measurement.

To overcome these difficulties a small amount of transferrin was added to the pulsed cytochrome oxidase as an internal standard. Transferrin is an iron-containing protein (Aasa *et al.*, 1963) displaying a strong e.p.r. signal at $g = 4.2$ and a weaker one at $g = 9$, lines that are well spaced and do not interfere too much with the cytochrome oxidase e.p.r. signals. However, when cyanide was added to the cytochrome oxidase solution, the transferrin signal at $g = 4.2$ overlayed the $g = 3.55$ cyanide signal, which made quantification difficult. Fortunately, though, the transferrin signal could be subtracted digitally without disturbing the rest of the e.p.r. spectrum. The amplitude of the $g = 4.2$ signal of transferrin added to the samples was measured, and this value was used as an internal standard to correct the ensuing integrations of the e.p.r. signals of the samples for small variations in sample temperature, instrumental sensitivity and small differences in packing between samples. These integrations were made by the procedure of Aasa & Vänngård (1975), except for the cytochrome *a* signal, for which instead the amplitude of the $g_x = 1.45$ signal was measured relative to the oxidized control sample. The *g* values for the low-spin a_3–HCN signal was calculated by the method of De Vries & Albracht (1979).

Results and discussion

Rapid-freeze experiments

Figs. 1 and 2 show e.p.r. spectra of samples obtained from experiments in which cytochrome *c* oxidase, as either the resting (Fig. 1) or the pulsed (Fig. 2) form, was mixed with its substrates, ferrocytochrome *c* and O_2, in the presence or in the absence of KCN, and then quenched by the rapid-freeze technique. As a comparison and for quantitative analysis each Figure shows a control experiment in which the enzyme, again in either the resting or the pulsed form, was quenched in the absence of cytochrome *c*. These Figures also show spectra of samples captured either directly following mixing (no delay) or 5s after mixing.

The signals seen in these spectra are typical of the enzyme and have been reported elsewhere (Aasa *et al.*, 1976; Johnson *et al.*, 1981). Thus the $g = 3$ (low-spin ferric cytochrome *a*) and $g = 2$ (cupric Cu_A) signals are seen in the controls and, to various extents, in the enzyme after mixing with substrates. In the experimental samples ferricytochrome *c*, produced as a consequence of electron transfer to the enzyme, makes the dominant contribution to the $g = 3$ region. In addition, the $g = 6$ signal of high-spin ferric cytochrome a_3 appears in some of the spectra of the partially reduced enzyme.

One of the important results of the experiments may be readily appreciated by a qualitative inspection of Figs. 1 and 2. It is seen that for both resting and pulsed enzyme the addition of cyanide makes very little difference to the e.p.r. signals displayed by samples frozen at very short times after mixing, i.e. the general features of spectra *B*

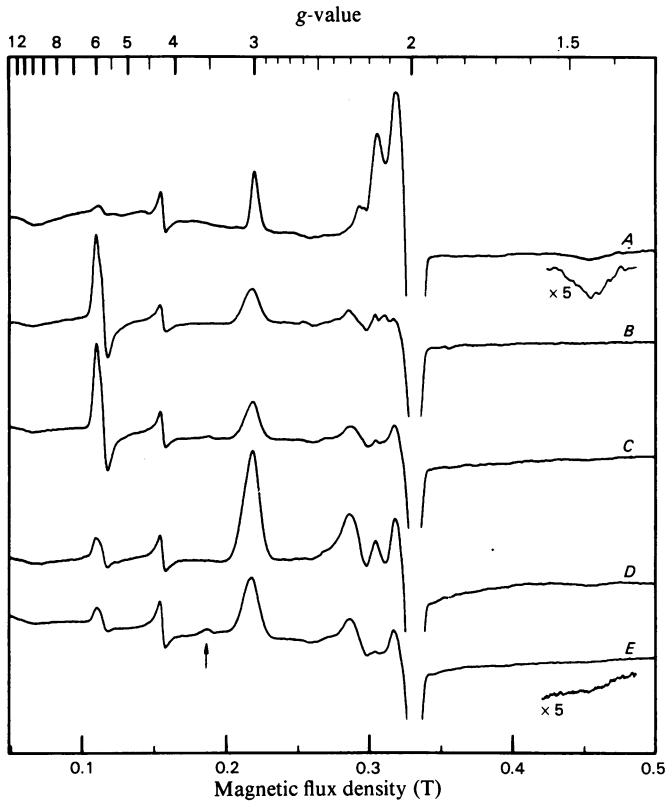

Fig. 1. *E.p.r. spectra from rapid-freeze experiments with resting oxidized cytochrome oxidase versus reduced cytochrome c*

Spectrum *A*: resting control sample, 123 μM-cytochrome oxidase (after mixing with Hepes buffer). Spectrum *B*: cytochrome oxidase mixed with cytochrome c^{2+}; no delay after mixing; cytochrome oxidase concentration, 123 μM; cytochrome *c* concentration, 1 mM. Spectrum *C*: as for spectrum *B* but with 10 mM-KCN (added in the cytochrome *c* syringe). Spectrum *D*: as for spectrum *B* but with 5 s delay after mixing. Spectrum *E*: as for spectrum *C* but with 5 s delay after mixing. All concentrations are after mixing. The arrow indicates the signal due to low-spin ferricytochrome a_3–cyanide complex at $g = 3.55$. The magnified signals at g_x 1.45 is due to cytochrome *a*. E.p.r. conditions: microwave power, 2 mW; microwave frequency, 9.222 GHz; microwave modulation, 2 mT; temperature, 15 K. The gain was the same for all spectra.

and *C* of Fig. 1 are similar, as are those of spectra *B* and *C* of Fig. 2 (see also the quantification below). In contrast, however, the spectra of samples frozen 5 s after mixing are significantly different in the absence and in the presence of cyanide (compare spectra *D* and *E* of each Figure). The $g = 3$ signal, due to the production of ferricytochrome *c* by aerobic oxidation catalysed by the enzyme, is much smaller in the presence of cyanide. It is apparent therefore that, during the first 5 s after mixing, cyanide inhibition is fully established. Indeed, examinaton of the e.p.r. spectra (not shown) of samples captured 1 s after mixing shows that in the presence of cyanide the $g = 3$ signal due to ferricytochrome *c* is developed to its maximum extent and does not increase thereafter. Thus the influence of cyanide is developed within the first second of turnover.

A quantitative analysis of these spectra may be obtained by integrating the signals as described in the Materials and methods section. The results of this quantification are shown in Table 1 and are now discussed below.

Resting enzyme

For the resting enzyme and within the dead time of the apparatus approx. 1.4 electron equivalents of ferricytochrome *c* are produced both in the absence and in the presence of 10 mM-cyanide. This is to be expected, as it is known that a rapid burst phase of the reaction between ferrocytochrome *c* and cytochrome oxidase transfers 1–2 electron equivalents

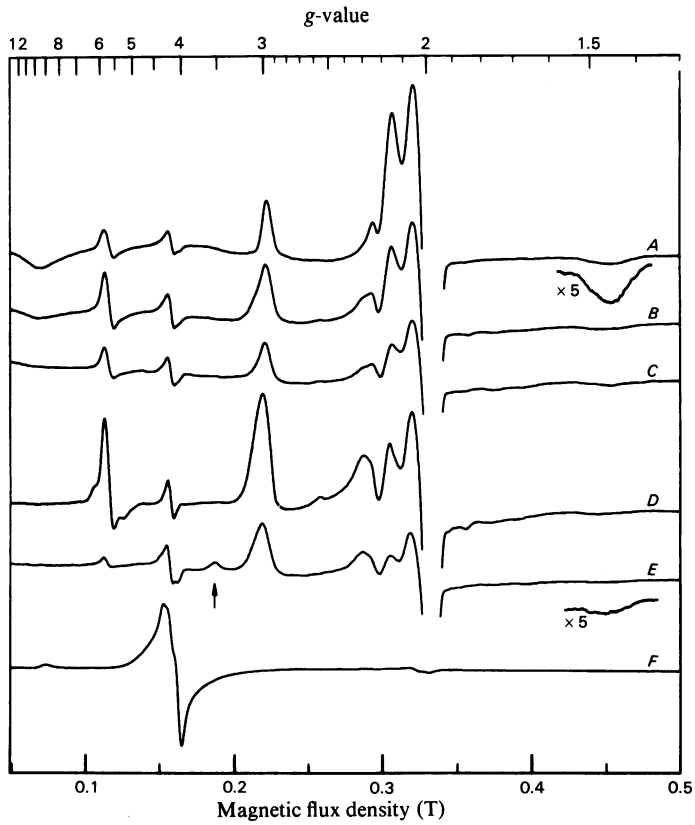

Fig. 2. *E.p.r. spectra from rapid-freeze experiments with the pulsed cytochrome oxidase versus reduced cytochrome c plus* O_2
Spectrum *A*: pulsed control sample, reduced cytochrome oxidase mixed with O_2-bubbled Hepes buffer and frozen without delay; cytochrome oxidase concentration, 173 μM after mixing. Spectrum *B*: reduced cytochrome oxidase mixed with cytochrome c^{2+} and O_2; no delay after mixing; cytochrome oxidase concentration, 173 μM; cytochrome *c* concentration, 1.25 mM. Spectrum *C*: as for spectrum *B* but with 10 mM-KCN (added in the cytochrome *c* syringe). Spectrum *D*: as for spectrum *B* but with 5 s delay after mixing. Spectrum *E*: as for spectrum *C* but with 5 s delay after mixing. Spectrum *F*: transferrin, 100 μM (internal standard); the signal shown was digitally subtracted from spectra *A*–*E*. Conditions were as described for Fig. 1. Freeze-dried transferrin was added to the cytochrome oxidase-containing syringe just before the start of the experiment.

Table 1. *Electron balance sheet for the reaction between ferrocytochrome c and cytochrome c oxidase*
The cytochrome c^{3+} values have been corrected for remaining cytochrome a^{3+}, i.e. cytochrome c^{3+} = total $g = 3$ signal − oxidized cytochrome *a*. * indicates that the electron distribution was unbalanced owing to transfer to O_2. — indicates that the signal was too small to integrate.

		Electron equivalents										
		No cyanide					With cyanide					
	Delay (s)	c^{3+}	a^{2+}	Cu_A^+	Cu_B^+ ($g = 6$)	Total (e⁻ in oxidase)	c^{3+}	a^{2+}	Cu_A^+	Cu_B^+	a_3^{3+}–HCN ($g = 3.55$)	Total (e⁻ in oxidase)
Resting	No	1.4	>0.9	0.75	0.2	1.85	1.4	>0.9	0.7	0.15	0.05	1.8
	5	4.5	0.7	0.7	—	*	2.4	>0.9	0.75	—	0.2	1.85
Pulsed	No	1.85	0.6	0.45	0.05	1.1	0.65	0.6	0.7	—	—	1.3
	5	4.8	0.7	0.4	0.13	*	2.05	0.80	0.65	—	0.3	1.75

from the donor in the first few milliseconds of the reaction and that this burst is insensitive to the presence of cyanide (Wilson *et al.*, 1975). The electrons from cytochrome *c* are distributed among the sites in the oxidase as indicated in Table 1. In each case cytochrome *a* is almost completely reduced and Cu_A is approximately 70% reduced. A small axial $g = 6$ signal arises within this time, this presumably being due to reduction of a portion of the Cu_B sites and thus the appearance of uncoupled ferricytochrome a_3. In the presence of cyanide a part of this latter signal is converted into $g = 3.55$, a low-spin ferric haem-cyanide signal. The total number of electron equivalents within the oxidase is approx. 1.8, somewhat higher than the expected value of 1.4 but probably equal to this value within the error of all the integrations involved.

After a 5s delay before quenching, the intensities of the e.p.r. signals are now quite different in the absence and in the presence of cyanide. In the former case the enzyme has clearly 'turned over' and catalysed the oxidation of cytochrome *c* by O_2. Thus the ferricytochrome *c* concentration is approx. 4.5 electron equivalents and approaches the maximum expected for this experimental regime. Under these conditions (1 mM-cytochrome *c* and 123 μM-oxidase) this maximum should be achieved when the O_2 is exhausted; assuming approximately 200 μM-O_2, we should see at maximum approx. 6 haem equivalents of cytochrome *c* oxidized (i.e. $200 \times 4 \div 123$), and less if the O_2 concentration is lower than 200 μM in the strong protein solutions used before mixing. The electrons cannot be accounted for by reduction of the sites within the enzyme and have presumably been passed on to O_2. In the presence of cyanide, however, only 2.4 equivalents of ferricytochrome *c* are seen: 1.9 of these are accounted for by the enzyme signals, whereas the remainder presumably have been passed to O_2 before complete inhibition ensued. The $g = 6$ signal has now vanished, to be entirely replaced by the cyanide-bound signal at $g = 3.55$, which is equivalent to 0.2 haem equivalent. This latter value is particularly interesting as it indicates that, although the enzyme is apparently fully inhibited, only 20% of cytochrome a_3 is in the low-spin ferric haem-cyanide complex and thus it cannot be this species that is of primary importance for the inhibition. Whatever the inhibited form is, it is seemingly e.p.r.-silent. A good candidate for this form is thus one in which both Cu_B and cytochrome a_3 remain oxidized and coupled, possibly by cyanide acting as bridging ligand, as suggested by Hill *et al.* (1983).

Our results on the resting enzyme are thus consistent with the following interpretations. On mixing the oxidized enzyme with ferrocytochrome *c* in the presence of cyanide (which does not bind to the oxidized enzyme on this time scale) approx. 2 electron equivalents enter 80% of the enzyme molecules. On achieving this state of partial reduction a transition occurs within the enzyme that allows cyanide to approach and bind to the binuclear cytochrome a_3–Cu_B site. In so doing, cyanide strongly stabilizes the oxidized forms of the metals to which it is tightly bound, thus stopping reduction of these sites and hence catalysis. In some 20% of the molecules, however, electrons may transfer also on to Cu_B, and the uncoupled ferricytochrome a_3 now becomes e.p.r.-visible and binds cyanide, again leading to inhibition. This interpretation thus supports the earlier kinetic studies which suggest that it is partially-reduced turnover intermediates that rapidly bind cyanide and also highlights the known heterogeneity of the resting enzyme.

Pulsed enzyme

The pulsed enzyme behaved in an essentially similar manner to the resting enzyme. In the absence of cyanide approx. 2 electron equivalents are delivered to the enzyme in the burst phase, as seen by the appearance of the ferricytochrome *c* e.p.r. signal. Half of these electron equivalents can be seen in the enzyme, the remainder we assume having passed into O_2. This situation is slightly different from that of the resting enzyme, probably reflecting the enhanced activity of the enzyme that the pulsed form is known to possess (Antonini *et al.*, 1977). After 5s the enzyme has catalysed the oxidation of the remaining cytochrome *c*.

In the presence of cyanide the situation is a little more complex. After 5s delay the pattern seen for the resting enzyme is repeated. Two electron equivalents have passed into the enzyme, which is now fully inhibited by cyanide, although only a fraction of the enzyme displays the $g = 3.55$ signal. With no delay, however, the picture is somewhat different. A significantly smaller amount of ferricytochrome *c* is formed (approx. 0.7 electron equivalent) while almost twice the electron equivalents are observed in the oxidase. We rationalize this situation as follows. Immediately after the mixing the enzyme for the pulsed experiments is still fully reduced in the presence of O_2. O_2 will now bind and accept 2 electron equivalents with a rate in the order of $3 \times 10^4 s^{-1}$. The sites in the enzyme donating these have until recently been considered to be cytochrome a_3 and Cu_B; however, Hill & Greenwood (1984) have shown that in a proportion of the molecules (40%) cytochromes *a* and a_3 may be the donors. In either case the enzyme will now exist with cytochrome a_3 and possibly Cu_B oxidized. Further electron transfer from Cu_A and cytochrome *a* to the O_2-binding site is slower, with a rate of approx. $5 \times 10^3 s^{-1}$, and

Table 2. *Electron equivalents remaining in cytochrome a and Cu_A*

Reduced cytochrome oxidase (246 μM) was mixed with aerated 50 mM-Hepes buffer, pH 7.4, at room temperature, and freeze-quenched in cold isopentane. The sample without cyanide (0 mM) was used as the reference and the e.p.r. signals from the completely re-oxidized cytochrome *a* and Cu_A in this sample were measured (see the Materials and methods section). The e.p.r. signals obtained after mixing the cytochrome oxidase solution with cyanide-containing aerated buffer (10 mM- and 30 mM-cyanide) were smaller than in the reference, and this difference was taken as a measure of the number of electron equivalents remaining in those samples.

Concn. of CN^- (mM)	Electron equivalents	
	a^{2+}	Cu_A^+
0	0	0
10	0.2	0.15
30	0.4	0.4

which is therefore comparable with the rate of cyanide binding. At 10 mM-cyanide and taking a rate constant of approx. $10^6\ M^{-1}\cdot s^{-1}$ for the second-order combination rate constant of cyanide to the partially reduced enzyme (Jones *et al.*, 1984), the cyanide 'on' rate is approx. 10^3–$10^4\ s^{-1}$. Thus there may be competition between cyanide binding to and reduction of the oxidized sites in cytochrome oxidase. If this is so, then we may expect that in a fraction of the molecules cyanide will bind to the enzyme and stop electron transfer from cytochrome *a* and Cu_A. The electrons that we observe on cytochrome *a* and Cu_A (Table 1, row 3: pulsed, no delay, plus cyanide) are therefore only partially derived from cytochrome *c* and are in fact electrons that have remained in the enzyme from the original reduction before the mixing. This also explains why we see more electron equivalents in the enzyme than we see cytochrome *c* oxidized.

Support for this explanation comes from control rapid-freeze experiments in which the fully reduced enzyme was mixed with oxygenated buffer either in the absence or in the presence of cyanide. Table 2 shows that in the absence of cyanide all the sites are oxidized within the dead time of the apparatus. In the presence of cyanide, however, the oxidation of cytochrome *a* and Cu_A is partially blocked, and this effect is exaggerated at higher cyanide concentrations. Such observations are consistent with the kinetic competition for sites as discussed above.

Reduction of cytochrome c oxidase by dithionite in the presence and in the absence of cyanide

Parallel experiments were carried out monitoring the reduction of cytochrome oxidase by dithionite either by optical or e.p.r. spectroscopic methods.

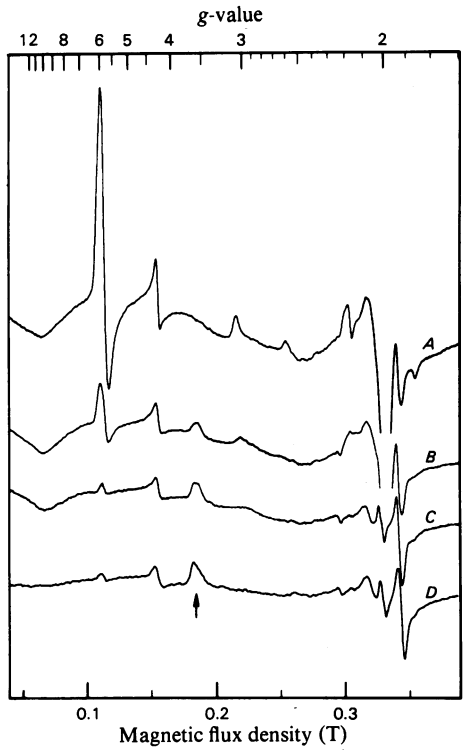

Fig. 3. *E.p.r. spectra of cytochrome oxidase after freeze–thaw experiments with cyanide present*

Spectrum *A*: cytochrome oxidase, 220 μM, frozen 10 s after the addition of $Na_2S_2O_4$ (6.5 mM) by immersing the sample in cold (140 K) isopentane ('fast freeze'). Spectrum *B*: as for spectrum *A* but with KCN (0.5 mM) added together with the dithionite. Spectrum *C*: the sample used for spectrum *B* (above) thawed to room temperature for 1 min and re-frozen ('fast freeze'). Spectrum *D*: the sample used for spectrum *C* after an additional 10 min thawing. Conditions were as described for Fig. 1.

At a wavelength of 445 nm and on addition of dithionite (25 mM) a rapid reduction of a portion of the enzyme (cytochrome *a*) was followed by slow reduction of cytochrome a_3 (Jones *et al.*, 1983) with a $t_{\frac{1}{2}}$ of approx. 150 s. In the presence of 300 μM-cyanide the rapid phase of reduction remained unchanged, but the rate of the slower phase was greatly lowered, $t_{\frac{1}{2}}$ approx. 1500 s. These results are in agreement with those reported by Jones *et al.* (1984) and show that cyanide binds rapidly to the partially reduced enzyme, stopping its full reduction. The rate of reduction of cytochrome a_3 is now limited by the cyanide 'off' rate.

Fig. 3 reports the complementary e.p.r. spectra.

Spectrum *A* is that obtained from an anaerobic (N_2-equilibrated) sample of resting oxidase into which 6.5mM-$Na_2S_2O_4$ was mixed and the same frozen after 10s. By this time the optical spectrum indicates that cytochrome *a* is largely reduced whereas cytochrome a_3 is oxidized. The e.p.r. spectrum (*A*) is in agreement with the optical measurements, the $g = 3$ signal (cytochrome *a*) is equivalent to only approx. 0.1 haem equivalent and the Cu_A is similarly small. The $g = 6$ signal (ferric cytochrome a_3) comprises about 0.2 haem equivalent and the remainder of the cytochrome a_3 is presumably still oxidized and coupled to Cu_B. Spectrum *B* shows the same experiment carried out in the presence of 0.5mM-KCN. Although optical (see above) and activity (Jones *et al.*, 1984) measurements suggest that the enzyme is now fully complexed with cyanide, we observe a $g = 3.55$ signal (cytochrome a_3^{3+}–HCN) comprising only about 0.15 haem equivalent, and the $g = 6$ signal has decreased by the same amount. On waiting for a further period (spectrum *C*), no additional signals appear, but the remaining $g = 6$ signal is converted into $g = 3.55$. The elapse of further time leads to no changes except that the character of the $g = 3.55$ signal is altered and the $g = 12$ signal disappears. Although we are at present uncertain of the significance of these changes, the observations are in agreement with those made by Johnson *et al.* (1981) and Brudvig *et al.* (1981), and we do not believe they alter our overall conclusions. The results of the optical and e.p.r. studies on dithionite reduction are therefore in full agreement with the rapid-freeze investigation with the native reductant, cytochrome *c*, namely that cyanide binding and inhibition gives an e.p.r.-silent species possibly involving binding to coupled ferricytochrome a_3 and cupric Cu_B. The heterogeneity observed in the rapid-freeze experiments is again evident in Fig. 3, with about 20% of the enzyme rapidly accepting electrons into Cu_B, rendering cytochrome a_3 e.p.r.-visible. This latter then binds cyanide to yield the $g = 3.55$ signal.

Concluding remarks

The behaviour of cytochrome a_3 (classically defined as the binding site of the oxidase) towards ligands is clearly strongly dependent not only on its valence state but also on the valence state of other metal centres and on the conformational status of the enzyme. When the fully reduced enzyme reacts with ligands, e.g. CO, CN, it does so in reactions that are second-order and whose equilibrium association constants measured by static titrations are in accord with those calculated from determinations of the 'on' and 'off' rates. In other words the ligand-binding site is accessible as it is in open and rapid communication with the bulk-phase solution. The same is true also for O_2, which binds exceedingly rapidly ($k_{on} \sim 10^8 M^{-1} \cdot s^{-1}$) to the cytochrome a_3–Cu_B binuclear centre.

Ligand binding to the fully oxidized enzyme is, in contrast, complex. For example, although cyanide binds very tightly to cytochrome a_3 ($K_D \sim 10^{-6}$ M), it does so very slowly and with a rate independent of cyanide concentration and with a high temperature coefficient. Such behaviour suggests that the binding site is shielded from approaching ligands and that complex-formation is rate-limited by transitions in the protein, possibly conformational, as suggested by the high enthalpy of activation (Van Buuren *et al.*, 1972), which allow ligands from the bulk phase to approach the binding site.

The transition from a 'closed' to an 'open' site is particularly important to the consideration of cyanide inhibition. It has been shown (Jones *et al.*, 1984; Van Buuren, 1972) that it is binding of cyanide to partially reduced forms of the enzyme, populated during turnover, that is responsible for the powerful inhibiting effects of cyanide. In these forms of the enzyme it appears that cytochrome a_3 remains oxidized yet is accessible to cyanide, as indicated by the rapid onset of inhibition (Antonini *et al.*, 1971). In a recent study (Jones *et al.*, 1984) a model was proposed that incorporated this transition from a 'closed' to an 'open' form on partial reduction. However, it was impossible to ascertain from the kinetic experiments the degree of reduction necessary to initiate this transition. The present study suggests that the entry of 2 electron equivalents into the enzyme from cytochrome *c* is sufficient to switch the cytochrome a_3 site to the accessible form. These electrons reside on cytochrome *a* and Cu_A, and thus it appears that reduction of the electron-accepting site is communicated to the ligand-binding site, which is now able to bind cyanide rapidly. The e.p.r. measurements also show that in the cyanide-bound form (apart from in a small fraction of the enzyme) cytochrome a_3^{3+} is still coupled to Cu_B^{2+}, and it is likely that the ligand is stabilizing both metals in their oxidized states by bridging between them, as suggested by Hill *et al.* (1983).

Although entry of 2 electron equivalents is sufficient to trigger the 'opening' of the ligand-binding site, we cannot be certain that both are necessary. The time-resolution of our method does not allow us to discount the possibility that reduction of one of the sites, cytochrome *a* or Cu_A, is sufficient.

In contrast, a common view has been that an input of 3 electron equivalents is necessary for cyanide binding (see, e.g., Nicholls, 1983). This divergence can, however, be explained by consid-

ering the different proportions of enzyme forms, by using the criteria of Brudvig *et al.* (1981), in oxidase prepared by the methods of Yonetani and Van Buuren (Wilson *et al.*, 1982) respectively. These differences are also reflected in the size of the $g = 3.55$ e.p.r. signal from the a_3^{3+}–HCN compex, which amounts to the equivalent of 1 haem with a Yonetani preparation (Johnson *et al.*, 1981) whereas with an enzyme preparation by the method of Van Buuren only about 20% of the molecules are in the form giving a $g = 3.55$ signal.

The nature of the transition leading to an open configuration may involve gross conformational changes [as suggested by the occluded nature of the resting site (see above) and by the change in the packing characteristics in the rapid-freeze experiments (see the Materials and methods section)], a displacement of an intrinsic ligand from the sixth co-ordination site of the haem of cytochrome a_3, or most probably both of these changes. For example fully oxidized pulsed enzyme, though being much more accessible to cyanide than the resting form (Brittain & Greenwood, 1976; Jones *et al.*, 1984), is certainly not as open to attack by cyanide as are partially reduced turnover forms.

The 'opening' and 'closing' of the site to ligands presumably has a functional significance for the enzyme *in vivo*. An obvious possibility is that it constitutes a device whereby O_2 may only approach the binding site when two metal centres are reduced. Thus, once electrons have passed to both Cu_B and cytochrome a_3, the O_2 may bind and the first 2-electron reduction to form tightly bound peroxide may proceed. A second possibility is that such a conformational transition constitutes part of the energy transduction system, e.g. the mechanical part of a proton pump.

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