Biochem. J. (1978) **173**, 65–72
Printed in Great Britain

Binding of Ligands and Spectral Shifts in Cytochrome *c* Oxidase

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(*Received* 19 *September* 1977)

1. On addition of reductant (ascorbate plus *NNN'N'*-tetramethyl-*p*-phenylenediamine) to isolated cytochrome *c* oxidase (ox heart cytochrome aa_3), in the presence of the inhibitors azide or cyanide, an initial partially reduced species is formed with absorption peaks at 415nm, 445nm and 605nm, which slowly gives rise to the final 'half-reduced' species in whose spectrum the 415nm peak has disappeared and a new absorption is seen at 430–435nm. 2. In the absence of reductant, cyanide forms an initial complex with the enzyme with a spectrum similar to that of the uncombined form, which slowly changes into the 'low-spin' cyanide form with a peak at 432nm. Azide, in absence of reductant, shifts the Soret peak slightly, but the resulting complex, which is probably thermally 'mixed-spin', undergoes no further changes. 3. The Soret-peak shift of oxidized cytochrome a_3 which occurs on reduction of the enzyme in the presence of azide is accompanied by a concurrent blue shift of the ferrous cytochrome *a* peak from 605nm to 603nm. A partial blue shift of the α-peak occurs in the half-reduced sulphide-inhibited enzyme, and a complete blue shift is seen in the analogous complexes with alkyl sulphides [$a^{2+}a_3{}^{3+}$HSR compounds, where R = CH_3, C_2H_5 or $(CH_3)_2CH$]. 4. Analogous, albeit less readily decipherable, spectroscopic effects with the ligands imidazole and alkyl isocyanides suggest that on reduction of cytochrome *a* an interaction occurs between the two haem groups involving (i) a high- to low-spin change in cytochrome a_3, and after this, (ii) a change in the molecular environment of the cytochrome *a*. The latter effect, possibly a decrease in the hydrophobicity of the haem pocket, requires that the ligands on cytochrome a_3 have a bulky and partially hydrophobic character.

The significance of spectral changes, other than those attributable to oxidoreduction, in the mechanism of mammalian cytochrome *c* oxidase action, has been discussed since the discovery of the supposedly 'oxygenated' form by Okunuki and his co-workers (Sekuzu *et al.*, 1959). More recently, it has been suggested that such changes may be associated with conformational change that may be required for either catalytic action itself or the coupling of electron transfer to energy conservation (Wikström *et al.*, 1976; Wikström & Saari, 1976; Wilson *et al.*, 1973).

Ehrenberg & Yonetani (1961), on the basis of magnetic-susceptibility measurements, suggested that 'resting' (ferric) isolated cytochrome aa_3 contained one low-spin haem group (cytochrome *a*) and one haem group in a thermally mixed-spin state (cytochrome a_3). A mixed-spin model has also been used by Thompson *et al.* (1976). On the other hand, Tsudzuki & Okunuki (1969) found no evidence for thermal equilibrium between spin states, and Babcock and co-workers (Palmer *et al.*, 1976; Babcock *et al.*, 1976) interpret their magnetic-c.d. studies in terms of a fully high-spin character for cytochrome a_3 in the ferric ground state. This viewpoint is supported by some more recent susceptibility measurements (Falk *et al.*, 1977).

Nevertheless, it is clear that changes occur on oxidoreduction in addition to those directly attributable to the reduction or oxidation itself. In the fully oxidized state, the high-spin (or thermally mixed-spin) cytochrome a_3 haem group is not detectable by e.p.r., as is also the case with one of the two cupric copper atoms (van Gelder & Beinert, 1969; Hartzell & Beinert, 1974). On partial reduction of the enzyme, high-spin ferric haem signals of several types ($g = 6.0$) are seen (van Gelder & Beinert, 1969; Hartzell & Beinert, 1976), indicating the presence of a species other than those found in fully oxidized and fully reduced states. When partial reduction is brought about in the presence of the terminal ligands formate, cyanide, azide and sulphide, which presumably all give rise to complexes of the form $a^{2+}a_3{}^{3+}$I, where I is the inhibitory ligand, signals characteristic of the $a_3{}^{3+}$I species are seen, both of the low-spin type ($g = 2.90$ and 2.75 for azide, $g = 3.6$ for cyanide, and $g = 2.54$ for sulphide) and of the high-spin type ($g = 6.0$ for formate). These intermediates have been identified by e.p.r. (Wever *et al.*, 1975), and by magnetic c.d. (Babcock *et al.*, 1976) as

well as by conventional spectrophotometry (Nicholls *et al.*, 1976). The last paper raised questions as to the relations between the structures of the two spectroscopically detectable types of complex formed with inhibitors, the fully oxidized $a^{3+}a_3{}^{3+}$I type and the half-reduced $a^{2+}a_3{}^{3+}$I type.

The present experiments were carried out in an attempt to answer some of these questions about the relationships between the two haem groups in the partially reduced states, in both the presence and the absence of inhibitors. A shorter version of this work was presented at the 11th FEBS Meeting, Copenhagen (Nicholls & Hildebrandt, 1977).

Materials and Methods

Ox heart cytochrome *c* oxidase (cytochrome aa_3) was prepared by either the method of Kuboyama *et al.* (1972) or the method of van Buuren (1972). It was stored at −75°C in 100mM-sodium/potassium phosphate (pH7.4)/0.25% Tween 80 solution. Samples were rapidly frozen in liquid N_2 before being placed in the −75°C refrigerator.

Cytochrome *c* (type VI from horse heart), *NNN'N'*-tetramethyl-*p*-phenylenediamine (as dihydrochloride) and ascorbic acid (as sodium salt) were from Sigma Chemical Co., St. Louis, MO, U.S.A. NaN_3 (Sigma), sodium formate (BDH, Poole, Dorset, U.K.), Na_2S (BDH AnalaR), KCN (BDH), propane-1-thiol (Aldrich Co., Milwaukee, WI, U.S.A.), propane-2-thiol (Aldrich), ethanethiol (Pfaltz and Bauer, Stamford, CT, U.S.A.), methanethiol (Pfaltz and Bauer) and imidazole (Sigma) were among the inhibitors used. Methyl isocyanide and ethyl isocyanide were prepared as described by Casenova *et al.* (1963) from the corresponding formamides (Eastman Co., Rochester, NY, U.S.A.), and n-butyl isocyanide was obtained from Aldrich.

Spectral studies were carried out with an Aminco DW-2 instrument in double-wavelength or split-beam modes. O_2 uptake was monitored with a Yellow Springs Instruments oxygen electrode coupled via an appropriate circuit to a Perkin–Elmer recorder.

Results

Fig. 1 illustrates the behaviour of isolated cytochrome *c* oxidase on the addition of ascorbate plus *NNN'N'*-tetramethyl-*p*-phenylenediamine under aerobic conditions. Even in the absence of cytochrome *c*, this reductance system induces an immediate and almost complete reduction of cytochrome *a* measured at either 445nm or 605nm (Nicholls & Kimelberg, 1968). At the same time, measurements at 433nm minus 413nm indicate the occurrence of much slower changes after cytochrome *a* reduction. A red shift is taking place in the Soret band of the ferric haem, i.e. cytochrome a_3 [cf. Figs. 2(*a*) and 4(*a*) below]. As shown in Fig. 1(*a*), this shift takes place

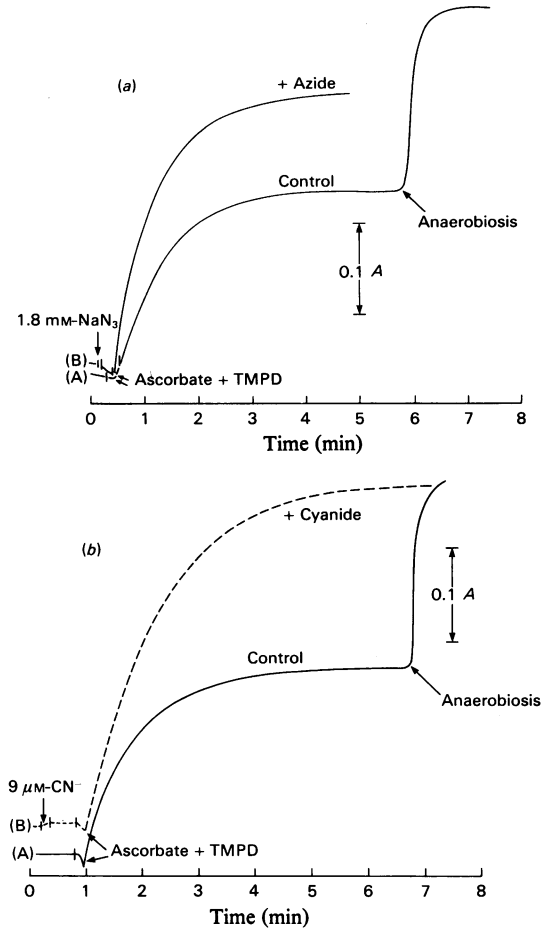


Fig. 1. *Changes in ferric cytochrome a_3 measured at 433−413nm on reduction of cytochrome a under aerobic conditions*

(*a*) Comparison of uninhibited system with the azide-inhibited system. The system contained 12mM-ascorbate+0.27mM-tetramethylphenylenediamine (TMPD) added to 3.54μM-cytochrome aa_3 in 67mM-sodium/potassium phosphate buffer+0.33% Tween 80 at pH7.4, 30°C. (A) control; (B) +1.8mM-NaN_3. (*b*) Comparison of uninhibited system with the cyanide-inhibited system; 9mM-ascorbate+0.27mM-tetramethylphenylenediamine were added to 3.4μM-cytochrome aa_3 in 100mM-sodium/potassium phosphate buffer+0.25% Tween 80 at pH7.25, 30°C. (A) ——, control; (B) ----, +9μM-KCN, added approx. 30s before reductant.

both with the free and presumably unliganded enzyme (trace A) and with the oxidized enzyme in the presence of azide (trace B), after the latter has apparently bound to the ferric enzyme (Wever *et al.*, 1973*a*). It also occurs with cyanide present (Fig. 1*b*), provided that sufficient time has not elapsed for cyanide

binding to the oxidized enzyme ($k \sim 2 M^{-1} \cdot s^{-1}$), which results in a similar spectroscopic shift in absence of reductant (van Buuren *et al.*, 1972). On the other hand the 433–413nm change does not occur in the presence of formate (Nicholls, 1976). When ascorbate plus *NNN'N'*-tetramethyl-*p*-phenylenediamine are added to a solution of cytochrome aa_3 in the presence of formate, therefore, the 'initial' (scanned at 10nm/s immediately after reduction) and 'final' (steady-state) spectra are essentially the same. Formate holds ferric cytochrome a_3 in an apparently high-spin state whether cytochrome *a* is reduced or oxidized.

Fig. 2 shows the spectra obtained initially and in the final steady state on addition of reductant to the uninhibited enzyme. The 'spin-state' shift apparently occurring in the Soret region is accompanied by a small shift in the α-peak with isosbestic point at 600nm and by a decline in the percentage reduction of cytochrome *a* measured at 445–465nm and 605–630nm. Similar changes have been reported by Okunuki (1966), but were analysed in a very different way (see below). In the presence of cyanide, a transition occurs even in the absence of reductant (van Buuren *et al.*, 1972). But if the reductant is added before the cyanide-induced shift, as in Fig. 1(*b*), the spectra given in Fig. 3 are obtained. In the Soret region an initial 'high-spin' complex, in which cytochrome *a* is fully reduced, is transformed into the usual cyanide-inhibited species ('cyanocytochrome aa_3') described previously (Nicholls *et al.*, 1972). The initial complex is catalytically inactive, yet shows the 415nm Soret band and 655nm shoulder (Hartzell & Beinert, 1976) of 'high-spin' cytochrome a_3.

When the analogous experiment is carried out in the presence of azide, a link is seen between the changes in the ferric species observable in the Soret region and the changes in the ferrous species in the α-region (Fig. 4). Azide forms a spectroscopic complex with fully oxidized enzyme characterized by rather small changes in the Soret and visible regions (Wever *et al.*, 1973*a*,*b*). Both kinetic and equilibrium characteristics of this $a^{3+}a_3{}^{3+}HN_3$ complex indicate that it may represent one of the species responsible for the inhibition by azide. However, the spectroscopic changes involved are small by comparison with those induced by cyanide, sulphide or formate (Nicholls *et al.*, 1976). Fig. 4 shows that the initial species produced after reduction of the $a^{3+}a_3{}^{3+}HN_3$ complex has a split Soret band at 415nm and 445nm, together with an α-band at 605nm. On standing, changes occur in the Soret region resembling those taking place in the uninhibited and cyanide-treated systems (Figs. 2 and 3). The 415nm ('high-spin' ferric) peak moves towards the red to give a complex similar to that with cyanide. At the same time the 605nm (ferrous) peak moves towards the blue to give the characteristic azide-induced α-peak shift (Nicholls & Kimelberg, 1968; Wilson & Chance, 1967).

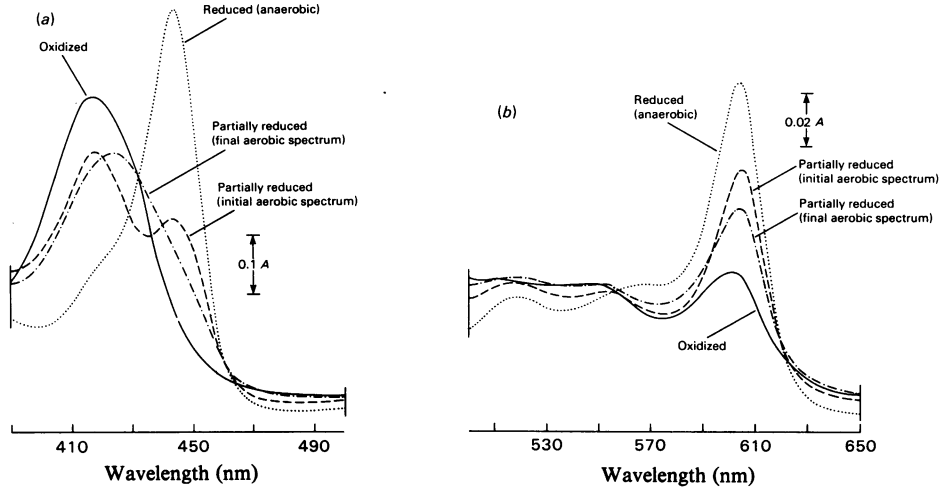


Fig. 2. *Spectra of cytochrome aa_3 in the early and late aerobic steady state induced by addition of ascorbate plus tetramethylphenylenediamine (TMPD)*

The system contained 3.0μM-cytochrome aa_3 in 50mM-sodium phosphate buffer + 0.25% Tween 80 at pH7.4, 30°C; 14.5mM-ascorbate + 0.27mM-TMPD were added as indicated. (*a*) Soret region (390–500nm); (*b*) visible region (500–650nm). ——, Oxidized enzyme before additions; – – –, partially reduced enzyme, spectrum scanned immediately after reduction; –·–·, partially reduced enzyme, spectrum scanned after reduction had reached a steady-state; ····, fully reduced enzyme after anaerobiosis.

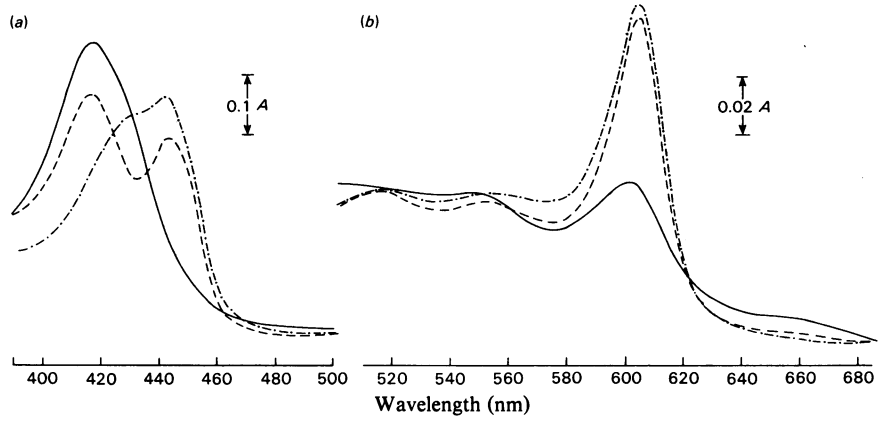


Fig. 3. *Spectra of cyanide-inhibited cytochrome aa_3 in the initial and final stages after addition of ascorbate plus tetramethylphenylenediamine (TMPD)*

The system contained 3.0 μM-cytochrome aa_3 in 100 mM-sodium phosphate buffer + 0.25% Tween 80 at pH 7.3, 30°C; 9.0 mM-ascorbate + 0.27 mM-TMPD + 1.8 mM-KCN were added as indicated. (*a*) Soret region (390–480 nm); (*b*) visible region (520–680 nm). ——, Oxidized enzyme before additions; – – – –, partially reduced enzyme, spectrum scanned immediately after reduction in the presence of cyanide; –·–·, partially reduced enzyme spectrum scanned in the final cyanide-inhibited state, 32 min after reduction.

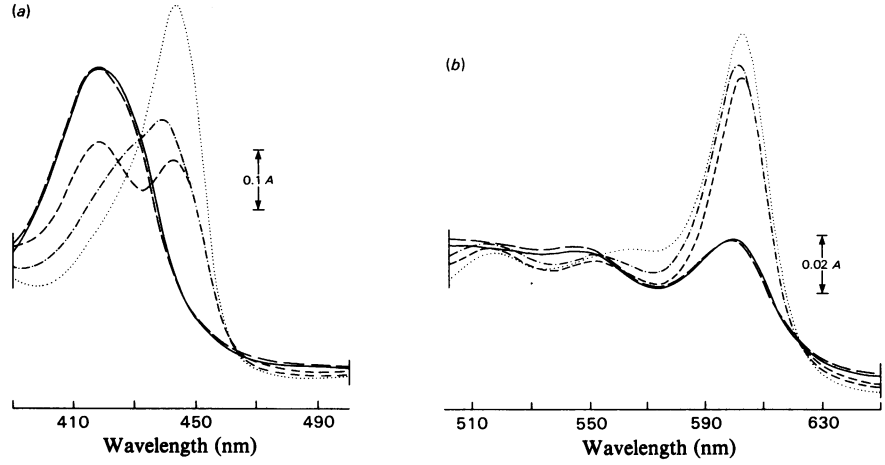


Fig. 4. *Spectra of azide-inhibited cytochrome aa_3 in the initial and final stages after addition of ascorbate plus tetramethylphenylenediamine (TMPD)*

The system contained 3.0 μM-cytochrome aa_3 in 50 mM-sodium phosphate buffer + 0.25% Tween 80 at pH 7.4, 30°C; 14.5 mM-ascorbate + 0.27 mM-TMPD + 3.6 mM-azide, were added as indicated. (*a*) Soret region (390–500 nm); (*b*) visible region (500–650 nm). ——, Oxidized enzyme before additions; — — —, oxidized enzyme + azide; – – – –, partially reduced enzyme, spectrum scanned immediately after reduction of azide complex; –·–·, partially reduced enzyme, spectrum scanned after inhibition and reduction had reached a steady-state; ····, fully reduced enzyme after anaerobiosis in the presence of inhibitor.

The blue shift of the α-peak is thus correlated with the Soret-band shift towards the red. Yet the α-peak shift does not occur with cyanide as ligand, even though, like azide but unlike formate, it gives a 'low-spin' (red-shifted) Soret peak (Fig. 3). Fig. 5 shows the difference spectra (anaerobic minus liganded forms) for cytochrome $a^{2+}a_3{}^{3+}$HSR complexes [where R = H, C_2H_5 or $(CH_3)_2CH$]. As previously reported, sulphide induces a partial α-peak shift (Nicholls, 1975), but the alkyl sulphides induce a

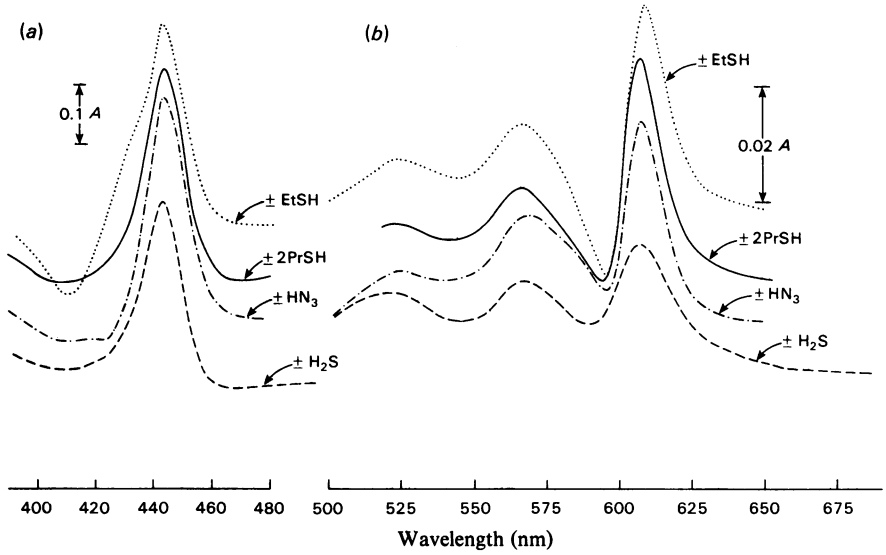


Fig. 5. *Difference spectra of 'half-reduced' complexes of cytochrome aa_3 with alkyl hydrogen sulphides*
The cuvettes contained 3.45 μM-cytochrome aa_3 in 50 mM-sodium phosphate, 0.5% Tween 80, pH 7.4, 30°C, reduced with 14.5 mM-ascorbate + 0.545 mM-tetramethylphenylenediamine. Sample cuvette was anaerobic; reference cuvette contained inhibitor as indicated: ----, 0.54 mM-$(NH_4)_2S$; -·-·, 5.5 mM-NaN_3; ——, 9 mM-2-propyl hydrogen sulphide; ····, 20 mM-ethyl hydrogen sulphide. (*a*) Soret region (390–480 nm); (*b*) visible region (500–650 nm).

shift essentially identical with that seen with the azide complex. Some ligand hydrophobicity seems to be required for the shift to be complete.

Transitions from one spin state to another may be expected for the unliganded system (Fig. 2) and for the azide-inhibited system (Fig. 4). Cyanide, however, might be expected to show a complex with a 430 nm peak at all times, just as the formate-inhibited system is always 'high-spin' (Nicholls, 1976). The initial complex seen in the steady-state experiments (Fig. 3) is not simply free enzyme. This would imply that cyanide binding was slow even at high cyanide concentrations, whereas kinetic observations show that the inhibition is in fact quite rapid (Nicholls *et al.*, 1972). Stoicheiometric quantities of cyanide are sufficient to block cytochrome oxidase activity completely (Fig. 6). On addition of reductant to cytochrome aa_3, the initial reduction measured at 447–460 nm is high, but declines as the oxidase is activated and the spin-state shift occurs (Figs. 1 and 2). Fig. 6 shows that cyanide addition increases the percentage reduction in the steady state after addition of ascorbate plus *NNN'N*-tetramethyl-*p*-phenylenediamine to isolated cytochrome aa_3. At saturating cyanide concentrations, the percentage reduction measured at 447–460 nm was 49% of that which takes place on anaerobiosis in the absence of inhibitor. As shown in Fig. 6, this degree of reduction is achieved by amounts of cyanide stoicheiometric with the cytochrome aa_3 present. At the same time the duration of the steady state increases with [HCN] until at stoicheiometric concentrations the enzyme is fully inhibited (curve □). At intermediate degrees of inhibition, not only is the steady-state reduction increased and the catalytic activity diminished, but the amount of enzyme reduced during the transition to anaerobiosis also decreases. Thus in the presence of 1.8 μM-HCN, anaerobiosis caused an absorbance increase at 447–460 nm of 0.255 (from 0.165 to 0.420) compared with an increase of 0.500 in the uninhibited system (from 0.060 to 0.560). These results are consistent with the model proposed previously (Nicholls *et al.*, 1972), involving a two-stage binding of cyanide to the enzyme, which is discussed further below.

Spin-state changes on ligand binding and/or reduction also take place in mitochondria and submitochondrial particles. In Keilin–Hartree submitochondrial particles (Keilin & Hartree, 1939), the 433–413 nm transitions (cf. Fig. 1) occur at rates somewhat higher than in the isolated enzyme. In both the presence and the absence of azide (cf. Fig. 1*a*), the observed rates were about fivefold faster ($k \sim 0.11\,s^{-1}$ rather than $0.02\,s^{-1}$ at pH 7.4, 30°C). The difficulty with reduction of the intact membrane system is the contribution in this region from cytochrome *b*, which antimycin addition does not completely eliminate. In a fully oxidized system, the cyanide reaction can be followed, as shown in Fig. 7. As with the isolated enzyme (Fig. 6), binding is

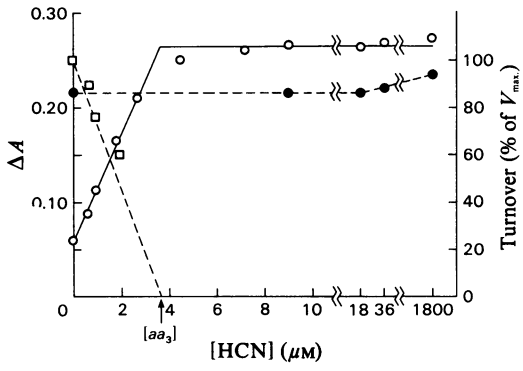


Fig. 6. *Reduction of cytochrome aa_3 and catalytic activity in the presence of increasing amounts of cyanide*
The cuvette contained 3.67 μM-cytochrome aa_3 in 100 mM-sodium phosphate/0.25% Tween 80, pH 7.23, 30°C, reduced with 9 mM-ascorbate + 0.27 mM-tetramethylphenylenediamine under aerobic conditions with cyanide additions as indicated. Reduction was measured at 447–460 nm (ΔA). ●, Reduction immediately after addition of reductant; ○, steady-state reduction; □, turnover (estimated from time taken to anaerobiosis at the lower cyanide concentrations, approaching infinity at [CN^-] > 3 μM). [aa_3] indicates concentration of cytochrome aa_3 present.

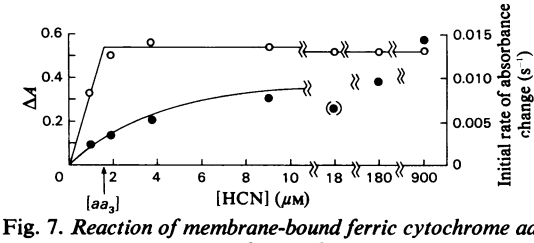


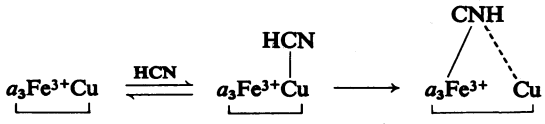
Fig. 7. *Reaction of membrane-bound ferric cytochrome aa_3 with cyanide*
Approx. 1.6 μM-cytochrome aa_3 (2.67 mg of submitochondrial particles previously treated with 1% deoxycholate/ml) were suspended in 100 mM-sodium phosphate, pH 7.5, with additions of cyanide as indicated. Reaction was measured at 433–413 nm (ΔA by dual-wavelength spectrophotometry). ○, Total absorbance change at cyanide concentrations indicated; ●, initial rate of absorbance change (s^{-1}) at cyanide concentrations indicated (cyanide binding and apparent associated spin-state transition). [aa_3] indicates concentration of cytochrome aa_3 present.

stoicheiometric between cytochrome aa_3 present and cyanide added. As shown by Erecinska *et al.* (1972) for mitochondria, the maximum rate for the high-spin to low-spin shift is similar to that seen with isolated enzyme, but the K_m (apparent) for the rate of the transition is much lower than for the isolated enzyme (van Buuren *et al.*, 1972). This is discussed further below. For the Keilin–Hartree submitochondrial particles, K_d for cyanide binding is less than 0.1 μM, whereas K_m (apparent) is about 5 μM.

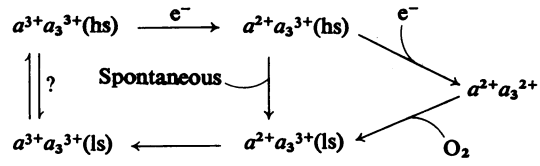
Discussion

The α-peak shift observed in the presence of azide has been a puzzle since it was first seen by Wilson & Chance (1967). Nicholls & Kimelberg (1968) attributed the shift to haem–haem interaction between cytochrome a^{2+} and cytochrome $a_3{}^{3+}HN_3$, whereas Wilson and his co-workers have favoured the idea that azide under some conditions may interact directly with the cytochrome a moiety in the enzyme. Azide may also interact with one of the enzyme copper atoms, with the latter mediating the observed interaction of the haem groups (cf. Yong & King, 1972). The present results show that if the complex seen with the fully oxidized enzyme is indeed an azide complex of the cytochrome a_3 haem group (Wever *et al.*, 1973*a*), then that complex can be partially reduced without inducing the α-peak blue shift. Only when the initial azide complex is transformed into its final state, with a red-shifted Soret peak, does the α-peak of ferrous cytochrome c move from 605 to 603 nm. The simplest hypothesis would predict that this shift was a function of the spin state of the cytochrome a_3 haem group, but that is readily falsified by the classical observation that the spectrum of the $a^{2+}a_3{}^{3+}$HCN complex has an α-peak in the same place as in that of $a^{2+}a_3{}^{2+}$ (Keilin & Hartree, 1939). The next most simple hypothesis would predict that this shift was a function not of the spin-state itself but of a spin-state change in the cytochrome a_3 haem group, from high-spin ($a^{3+}a_3{}^{3+}HN_3$) to low-spin ($a^{2+}a_3{}^{3+}HN_3$). But this is falsified by the observation that the spectrum of the $a^{2+}a_3{}^{3+}H_2S$ complex is partially blue-shifted, although $a_3{}^{3+}H_2S$ seems always to be 'low-spin' in character (Nicholls, 1975; Nicholls *et al.*, 1976).

The effect of alkyl sulphides in producing a shift analogous to that seen with azide (Fig. 5) suggests that the change requires both (i) a low-spin cytochrome a_3 haem group, and (ii) a somewhat lipophilic ligand to cytochrome a_3. The formate complex and the initial azide complex are high-spin, and show 605 nm peaks. The cyanide complex is low-spin, but the CN^- ion is small, and hence the peak remains at 605 nm. As the ligand size is increased, from SH^- through N_3^- to the SR^- compounds, so the peak shift increases. Preliminary experiments carried out with other potentially hydrophobic ligands have tended to confirm this. Isocyanide complexes with cytochrome aa_3 (cf. Yamamoto & Orii, 1973) show much higher shoulders at 590 nm than does the CO complex, suggesting that a shift in the cytochrome a peak may occur in addition to the formation of a complex of ferrous cytochrome a_3 with methyl isocyanide or ethyl isocyanide. Like CO, however,



Scheme 1. *Cyanide binding to cytochrome a_3 haem group*



Scheme 2. *Transformation of high-spin (hs) (418 nm Soret band) into low-spin (ls) (428 nm Soret band) species of cytochrome aa_3*

these reagents do not react with ferric haem, nor are the ferrous complexes autoxidizable.

Imidazole is another rather bulky inhibitor of cytochrome oxidase activity, and in the partially reduced steady state the α-peak was found to be blue-shifted. The concentrations of imidazole required are, however, rather high (over 0.25 M), and irreversible denaturation of the enzyme with a complete peak shift to the 590 nm position characteristic of a haemochromogen complex of haem *a* occurs in this system.

As suggested previously (Nicholls *et al.*, 1976) the azide complex seen with the fully oxidized enzyme may be in a 'mixed-spin' state. When cytochrome *a* is reduced by ascorbate plus *NNN'N'*-tetramethyl-*p*-phenylenediamine, the cytochrome a_3 haem group is induced to change to a low-spin state; thereafter, the cytochrome a^{2+} haem group, sensing a low-spin configuration at the $a_3{}^{3+}$ centre, responds with an α-peak shift from 605 to 603 nm. If this interpretation is correct, and if Wever *et al.* (1973*a*,*b*) have correctly identified the $a^{3+}a_3{}^{3+}HN_3$ complex, then a problem arises about the nature of the cyanide complexes, as follows.

(i) Initial cyanide binding does not perturb the spectrum in either the ferric (van Buuren *et al.*, 1972) or half-reduced states (Fig. 3, above); (ii) initial cyanide binding is very sensitive to the state of the enzyme and oxidation–reduction of cytochrome *a*, and is promoted by the prior binding of azide (van Buuren *et al.*, 1972); (iii) the transition to the final cyanide complex is a slow reaction rather insensitive to the state of the enzyme, and involves a uniform tight and stoicheiometric binding (Fig. 6 above).

We conclude that there must be an initial binding site for cyanide, binding to which does not affect the spectrum and which is independent of the free or liganded state of cytochrome a_3. Such a site, which might be protein in nature, or involve one of the two Cu^{2+} components, would then be favourably oriented for the final transfer of ligand to the haem group, as in Scheme 1. Why the initial binding should show such great variability in apparent equilibrium (from 4 μM or less in mitochondria and submitochondrial particles, or in the half-reduced state, to 10 mM in the isolated ferric enzyme) is not known. At present it remains perhaps the most striking example of an 'allotopic' (Racker, 1971) property of cytochrome aa_3.

The relationship of these changes to the behaviour of the uninhibited enzyme is also uncertain. The transformation of 'high-spin' (418 nm Soret peak) into 'low-spin' (428 nm Soret peak) species was classically achieved by oxygenating the fully reduced species (Okunuki, 1966). Observations of a similar change in the presence of inhibitors suggest that the transformation depends more on the reduction of cytochrome *a* than the 'oxygenation' of cytochrome a_3. Scheme 2 illustrates the reactions envisaged here. Brittain & Greenwood (1976) have shown that cyanide binding to the 'oxygenated' (low-spin) form is in fact faster than to the ferric species as isolated.

The 'high-spin' (418 nm) form is thus probably the one seen in the isolated enzyme, with the e.p.r. characteristics reported by Falk *et al.* (1977). On reduction of the cytochrome *a* haem group, the half-reduced $a^{2+}a_3{}^{3+}$(hs) species is obtained, which is thermodynamically less stable than the $a^{2+}a_3{}^{3+}$ (ls) form. The latter can also be obtained by oxidizing the fully reduced form (Sekuzu *et al.*, 1959), and can be reoxidized to the fully oxidized species (428 nm peak), which can also be obtained from the $a^{3+}a_3{}^{2+}$ species on reaction with O_2. In the isolated enzyme, the $a^{3+}a_3{}^{3+}$ (ls) form is less stable than the $a^{3+}a_3{}^{3+}$ (hs) form, but in the membrane-bound form, the species with the 428 nm peak may persist.

Whether this tells us anything about the roles of these species in energy conservation (Wikström *et al.*, 1976) remains to be seen.

We thank Dr. John M. Wrigglesworth for discussions and Mr. G. A. Chanady for the preparations of alkyl isocyanides. This work was supported by grants A-0412 and E-4001 from the Canadian National Research Council to P. N.

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