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LIGAND-INDUCED SPECTRAL CHANGES IN CYTOCHROME *c* OXIDASE AND THEIR POSSIBLE SIGNIFICANCE

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SUMMARY

1. The spectral shifts induced on the binding of H_2S to ferric cytochrome aa_3 are similar to those induced by cyanide, reflecting a possible high- to low-spin state change in the a_3 haem. Opposite shifts are seen with either formate or low azide concentrations, while high azide concentrations reverse the change induced at lower concentrations. The unusually high Soret band in the half-reduced sulphide-inhibited species ($a^{2+}a_3{}^{3+}H_2S$) results from the superposition of cytochrome a^{2+} and cytochrome $a_3{}^{3+}H_2S$ peaks.

2. The difference spectra in the visible region for cytochrome a^{2+} minus cytochrome a^{3+} obtained with the four inhibitors (cytochrome $a^{2+}a^{3+}I$ minus $a^{3+}a_3{}^{3+}I$) are similar, except that azide and sulphide induce blue shifts of the α-peak. The trough in the Soret region for the azide complex is much deeper than that for the other complexes, suggesting changes in the cytochrome $a_3{}^{3+}HN_3$ centre on reduction of cytochrome *a*.

3. The "oxygenated" and "high-energy" forms of cytochrome aa_3 both involve spectral changes at the a_3 haem similar to the changes induced by cyanide and sulphide. The spectrum of partially reduced cytochrome aa_3 in the presence of reductant and oxygen indicates the steady-state occurrence of appreciable levels of low-spin (oxygenated) cytochrome aa_3. These may be important for energy conservation during the action of cytochrome aa_3 in the intact mitochondrial membrane.

INTRODUCTION

Perutz and his cowerkers [1–3] have shown that spin state changes in the haemoglobin iron atoms are linked to conformational changes in the protein moieties and are part of the haem-haem interaction system responsible for the characteristic sigmoid oxygen binding profile. Even methaemoglobin (ferric) iron spin state changes have conformational consequences [2, 3], although such changes are presumably not part of the normal haemoglobin reaction. Cytochrome *c* oxidase, which under-

Abbreviation: TMPD, *NN,N′,N′*-tetramethyl-*p*-phenylene diamine (as dihydrochloride).

goes a catalytic ferric-ferrous cycle, also shows spin state changes under such conditions [4–6]. Spectroscopic effects of a possibly related type are induced by energization [7] and have also implicated haem-haem interactions between the cytochrome *a* and a_3 components [8, 9].

Yet the usual model of cytochrome *c* oxidase in the inner mitochondrial membrane is that of a molecule penetrating the lipid bilayer [10, 11] whose function is solely to deliver electrons from cytochrome *c* on the outer side of the membrane to the oxygen molecule reduced on the inner side of that membrane. This model assigns no essential role to the observed spectroscopic and probable spin state changes which are assumed to be at most "electrochromic" responses in a system functionally no more complex than the ferrocene model of Hinkle [12]. Nor does it explain the complexities in the biosynthesis of cytochrome *c* oxidase [13, 14]. Both the biosynthetic picture and the chemiosmotic picture [15] of the oxidase need integrating with the "interacting dimer" and related mechanistic models [16, 17] that have been elaborated on the basis of functional and spectroscopic studies.

Following recent work with terminal inhibitors, both of the apparent low spin [18] and apparent high spin [19, 20] varieties, the present paper compares the changes seen with such inhibitors in the ferric and half-reduced states of isolated cytochrome aa_3 with the change induced by oxygenation of fully reduced enzyme [4]. It is suggested that such changes may be of functional importance to the operation of the oxidase molecule in situ and that the concepts of Perutz et al. [1–3] may be applicable to haemoproteins other than mammalian haemoglobin.

METHODS AND MATERIALS

Cytochrome *c* oxidase was prepared from beef hearts according to the method of van Buuren [21]. As recommended by the Amsterdam group, the preparation was made on a fairly large scale, commencing with over 12 kg minced muscle. In the presence of 1 % asolectin (Associated Concentrates Ltd., Woodside, L.I., N.Y.) the maximal turnover in 67 mM potassium phosphate, 0.5 % Tween-80, pH 7.4 at 27 °C was between 350 and 400 s^{-1} (electrons/s per aa_3 unit), measured in the presence of ascorbate and horse heart cytochrome *c* (Sigma type VI). The apparent K_m for cytochrome *c* is 12 μM. In the absence of asolectin, the turnover is about 80 % of that in its presence. As deoxycholate-treated submitochondrial particles assayed under similar conditions show a turnover of 450–500 s^{-1}, the enzyme as isolated is composed of at least 80 % active molecules [22, 23]. Moreover, unlike the previous preparation [18], the present material exhibited very little autoreduction, permitting the formation of the ferric complex with sulphide (Fig. 2, below).

Other methods and materials were as described previously [18, 20]. Spectra were obtained with a Cary 118 C instrument, and cytochrome aa_3 concentration determined using ΔE mM (605–630 nm), reduced minus oxidized equal to 27 (equivalent to 13.5 on a haem *a* basis).

RESULTS

Fig. 1 gives the difference spectra for four inhibitors of the terminal oxidase obtained with the fully oxidized enzyme (cytochrome $a^{3+}a_3{}^{3+}$). As indicated in

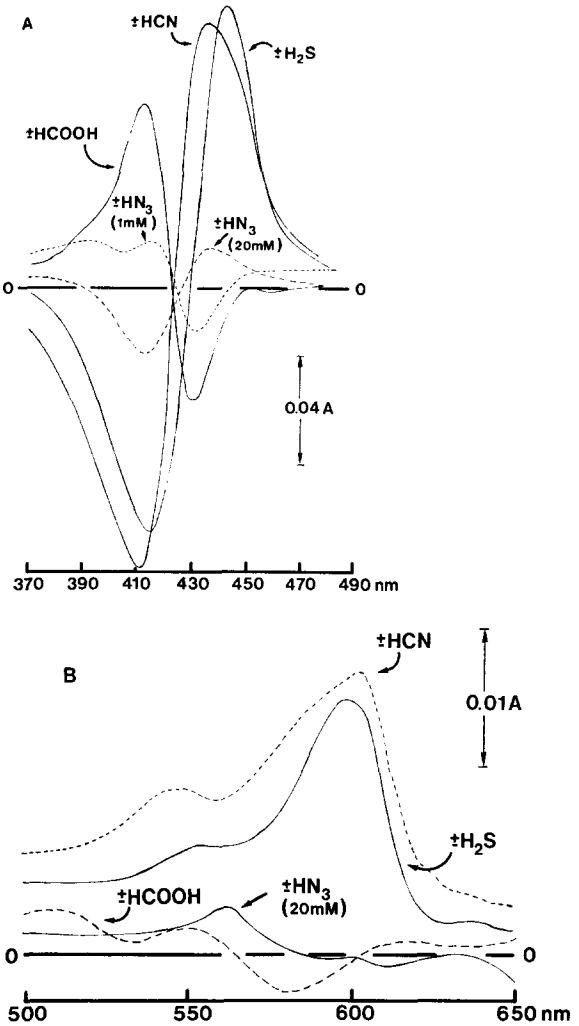

Fig. 1. Difference spectra of ferric cytochrome *c* oxidase in the presence and absence of the indicated inhibitors. A, Soret region. B, visible region (500–650 nm). 3.4 μM cytochrome aa_3 in 67 mM phosphate buffer pH 7.49, 0.5 %Tween-80, 27 °C, with the indicated inhibitor additions to the sample cuvette: 4.2 mM KCN, 48 μM Na_2S, 77 mM $HCOONH_4$, 20 mM NaN_3, and 1.1 mM NaN_3 (Soret region only), respectively.

Fig. 1A, formate induces a blue shift of the Soret band [19, 20], while cyanide creates a red shift with increasing absorption at 435 nm [24, 25]. By analogy with other haemoproteins [20] and by comparison with EPR [5, 6, 26] and magnetic susceptibility data [27], we suppose that the former represents a shift to a higher spin state and the latter a shift to a lower spin state. Sulphide has an effect similar to that of cyanide, but the red shift of the Soret peak is more marked with increased absorption occurring maximally at 445 nm, as shown in the sulphide difference spectrum given in Fig. 1A. This increase at 445 nm was previously seen in the partially reduced state [18] and attributed to partial reduction of the a_3 haem in the complex. Fig. 1B

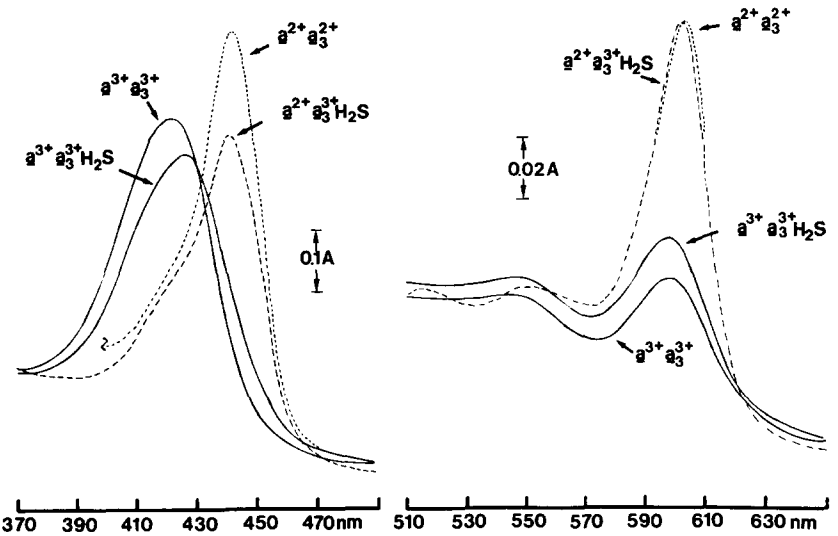

Fig. 2. Absolute spectra of sulphide complexes of ferric and half-reduced cytochrome *c* oxidase. 3.4 μM cytochrome aa_3 in 67 mM phosphate buffer, pH 7.4, 0.5 % Tween-80, 27 °C. ——, control; ——, plus 48 μM Na_2S; ····, anaerobic, plus 11.5 mM ascorbate and 230 μM TMPD; – – – –, aerobic steady state, plus 48 μM Na_2S, 11.5 mM ascorbate and 230 μM TMPD.

now renders the latter interpretation less likely, as the action of sulphide on the fully oxidized enzyme in the visible region is similar to that of cyanide, showing a broad band at 580–600 nm and a low β band at about 550 nm.

Fig. 2 gives the absolute spectra for the two sulphide complexes (cytochrome $a^{2+}a_3{}^{3+}H_2S$ and $a^{3+}a_3{}^{3+}H_2S$). The increased absorption between 440–450 nm in the fully oxidized complex is also reflected in the higher ferrous peak at 445 nm as compared with the cyanide complex [18]. In the visible region, the increased absorption at 580–600 nm is not seen clearly in the slightly blue-shifted α-peak of the half-reduced form. Evidently under these conditions the contributions of ferrous a_3 and of liganded ferric a_3 to the α-region spectrum are approximately equal.

As reported previously by Muijsers et al. [28], Fig. 1A shows that azide acts ambiguously. In its normal binding range (< 1 mM) the spectrum indicates a slight blue shift. At higher levels (20 mM) the Soret peak is shifted towards the red. Neither effect is as marked as those produced by the paradigm ligands, cyanide and formate, and the red shift effect is associated with only a small visible region band at 600 nm.

If inhibited enzyme is treated with reductant, only cytochrome *a* initially becomes ferrous. If (a) the spectrum of cytochrome *a* Fe^{2+} is independent of the ligand state of cytochrome a_3; and (b) the spectroscopic state of cytochrome a_3 complexes is independent of the redox state of cytochrome *a*, then the resulting spectra should be identical. Fig. 3 shows that this is not the case. The positive-going Soret (443-448 nm) and α (603–605 nm) peaks are of similar heights in the presence of the four inhibitors, but the α-peak is blue-shifted about 2.5 nm by azide and 1.0 nm by sulphide, and the Soret peak is blue-shifted by azide, as previously shown [29, 18]. But, in addition, the trough in the Soret region due to disappearance of oxidized enzyme

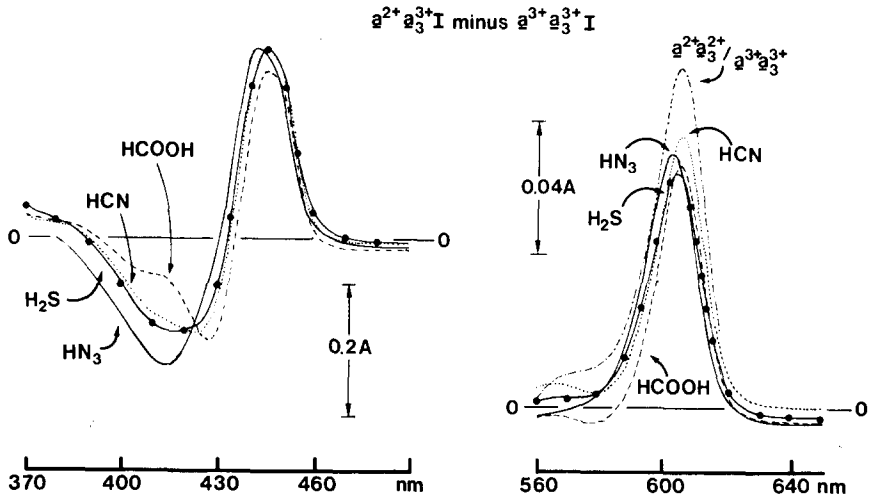

Fig. 3. Difference spectra of half-reduced cytochrome *c* oxidase in the presence of four inhibitors (cytochrome a^{2+} minus cytochrome a^{3+} associated with four types of liganded cytochrome $a_3{}^{3+}$). 3.4 μM cytochrome aa_3 in 67 mM phosphate buffer, pH 7.4, 0.5 % Tween-80, 27 °C. 11.5 mM ascorbate plus 230 μM TMPD added to sample cuvette. –·–·–, control, no inhibitor in sample cuvette (anaerobic minus oxidized spectrum); added to both reference and sample cuvettes: 4.2 mM KCN (····), 48 μM Na_2S (●—●), 77 mM $HCOONH_4$ (– – –), 20 mM NaN_3 (——).

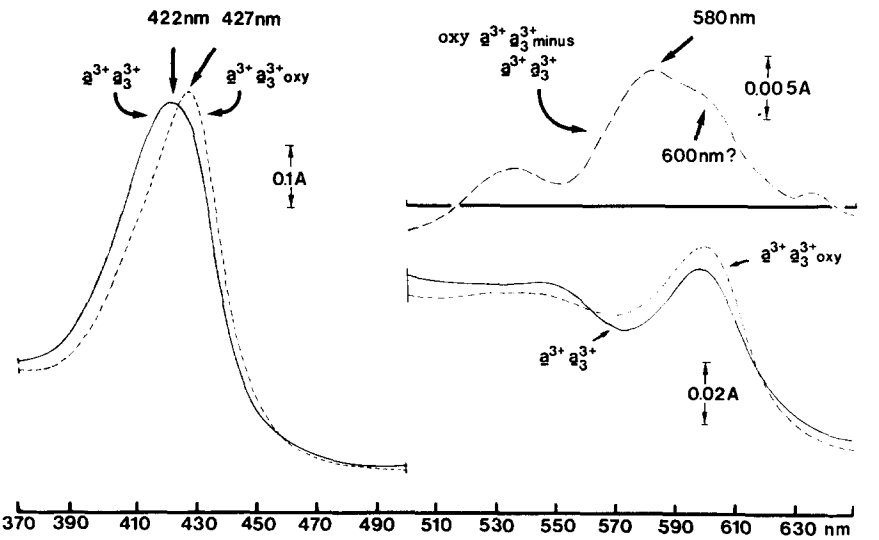

Fig. 4. Absolute and (visible) difference spectra of oxidized and oxygenated cytochrome *c* oxidase. 3.9 μM cytochrome aa_3 in 67 mM phosphate buffer, pH 7.49, 0.5 % Tween-80, 27 °C; ——, ferric enzyme (no additions); ----, enzyme reduced with dithionite and reoxidized by bubbling air ($a^{3+}a_3{}^{3+}$ oxy). (Inset: ———— ferric oxy minus normal ferric spectrum).

(cytochrome *a* Fe^{3+}) is different for the four inhibitors. Although cyanide and sulphide are similar (contrast Fig. 1A), the formate and azide effects are dissimilar. Azide induces the largest trough in the Soret region, although at both low and high concentrations it has the smallest spectroscopic effects on fully oxidized enzyme (Fig. 1A). Fluoride, reported as an oxidase inhibitor by Muijsers et al. [30], was also tried, but had very weak inhibitory action in our hands. Although a slight blue shift of the Soret band was seen with fully oxidized enzyme (not shown, cf. ref. 30), fluoride failed to block the system with added ascorbate plus TMPD, and partially reduced spectra were thus unobtainable, even after lowering the pH to 6.8 ($K_i > 100$ mM). Part of the reported inhibition [30] may be due to the action of fluoride on the reaction of cytochrome *c* with enzyme and not to its effect on intramolecular electron transfer.

To compare the spectra obtained with inhibitors and the spectra seen under catalytic conditions, the oxygenated complex (Fig. 4) was prepared by aerating a

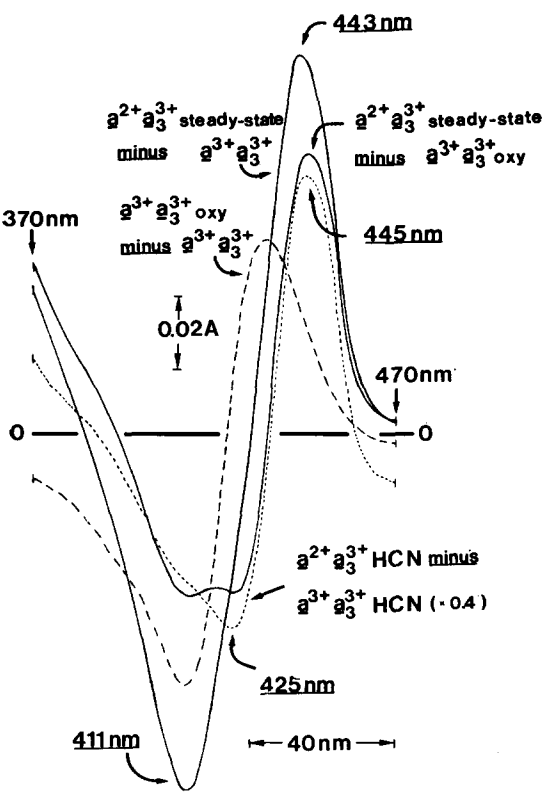

Fig. 5. Difference spectra of cytochrome *c* oxidase derivatives in the Soret region. 3.9 μM cytochrome aa_3 in 67 mM phosphate buffer, pH 7.49, 0.5 % Tween-80, 27 °C. ----, ferric oxy minus normal ferric spectrum (cf. visible spectra in Fig. 4); ——, steady-state spectrum with 11.5 mM ascorbate +385 μM TMPD minus normal ferric (maximum at 443 nm, minimum at 411 nm); ——, steady-state spectrum as above minus ferric oxy spectrum (maximum at 445 nm, minimum at 425 nm); ····, half-reduced cyanide-inhibited enzyme minus ferric cyanide complex spectrum (cf. Fig. 3) on 2/5 smaller scale.

dithionite-reduced enzyme sample according to Lemberg and Stanbury [4]. As seen in both Soret and visible regions (cf. inset difference spectrum) the product following oxidation by oxygen is spectroscopically very similar to complexes such as those formed with sulphide and cyanide. This similarity is documented more closely in Fig. 5. Here, four different spectra have been superimposed, all obtained with the same enzyme sample. A steady-state oxidation-reduction system was set up by treating the aerobic solution of cytochrome aa_3 with ascorbate and TMPD. At the concentration levels used, the cytochrome *a* was about 40 % reduced during the steady state. If the fully oxidized ($a^{3+}a_3{}^{3+}$) spectrum is subtracted from the steady state spectrum (full line, with 443 nm peak and 411 nm trough), the result is very different from the cytochrome *a* difference spectra seen in Fig. 3. But if the oxygenated (a^{3+} $a_3{}^{3+}$oxy) spectrum is subtracted instead (full line, with 445 nm peak and 425 nm trough), the result is closely similar to the reduced minus oxidized difference spectrum obtained in the presence of cyanide (dots). The cyanide spectrum is plotted here at a reduction of 60 % to allow for the fact that only 40 % of cytochrome *a* is reduced in the absence of inhibitors. Thus the aerobic steady state system in the absence of cytochrome *c* (cf. ref. 31) contains a form of ferric cytochrome a_3 (long dashes difference spectrum, Fig. 5) similar to that obtained by oxygenation of the dithionite-reduced enzyme (Fig. 4). In opposition to Lemberg's original view [4, 32], this further suggests that the oxygenated form involves only the cytochrome a_3 haem (cf. ref. 33).

DISCUSSION

Why does cytochrome *c* oxidase form such tight complexes with cyanide [24, 25] and sulphide [18]? Why are there such strong interactions between the *a* and a_3 haem groups [8, 34]? If the role of cytochrome *c* oxidase were merely to transfer electrons to the matrix side of the inner mitochondrial membrane, the former would seem dangerous and the latter redundant. If, on the other hand, conformational changes were essential to energy conservation at site III, and if such changes are linked to spin state changes as in haemoglobin [1–3], then cyanide and sulphide might be doing "abortively" what electron transfer [4] and energization [7] are doing functionally. That is, the unusually tight binding of haem ligands like cyanide and sulphide may reflect the tendency of the molecule to take up the alternative configuration under a variety of environmental stimuli.

One such environmental stimulus may be membrane energization [7]. The original chemiosmotic model suggested that the aa_3 system should respond only to the $\Delta\psi$ (membrane potential) and not to the ΔpH component of the proton motive force (cf. ref. 35). Such evidence as there is suggests that in fact the oxidase responds to both components; Erecinska et al. [36] report that each of the three sites of phosphorylation spans a similar redox gap, and Wikström [37] has found that the spectroscopic response of the oxidase can be brought about both by an appropriate ΔpH and by an appropriate $\Delta\psi$ across the mitochondrial membrane. These observations suggest that coupling between oxidase activity and energy conservation may be less direct than the simple chemiosmotic model required, and that intermediate conformational changes may be needed.

The present results also indicate further unusual features of the azide reaction

(cf. refs. 9, 26, 28). The difference spectra indicating haem-haem interaction (Fig. 3) suggest that while the cytochrome *a* Fe^{2+} spectrum is blue-shifted in both Soret and α-band regions, the spectroscopic state of the cytochrome $a_3{}^{3+}HN_3$ may also change as cytochrome *a* is reduced, from a blue-shifted (Fig. 1A, 1mM HN_3 spectrum) to a red-shifted state (cf. deep trough at 412 nm in Fig. 3). Even in the fully oxidized state, it seems that binding of a second azide molecule somewhere on the oxidase protein can alter the a_3 Fe^{3+}-HN_3 group from a state probably slightly more high-spin than the free species to one which is slightly more low-spin (cf. the 1 mM and 20 mM spectra in Fig. 1A, and see ref. 28). With other haemoproteins, the azide complex can exist in either a high or low spin form (cf. Table I in ref. 20). The cytochrome a_3 Fe^{3+} · azide complex may likewise be energetically poised between two spectroscopic or conformational states, while the cyanide and sulphide complexes are always in the red-shifted (low spin) configuration and the formate complex is always in the blue-shifted (high spin) form.

If the uninhibited enzyme, like the azide complex, can also exist in a variety of conformational states, perhaps linked to spin states as proposed for haemoglobin [1–3], then a search for a functional role for these states may prove fruitful. It is unlikely that the complex behaviour of the relatively recent haemoglobin molecule is determined by chemical interactions of a type not utilised at an earlier evolutionary stage.

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