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THE EFFECT OF SULPHIDE ON CYTOCHROME aa_3 ISOSTERIC AND ALLOSTERIC SHIFTS OF THE REDUCED α-PEAK

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SUMMARY

1. Sulphide, like cyanide, is a slow-binding inhibitor of cytochrome aa_3 with a high affinity ($K_d < 0.1$ μM).

2. Unlike cyanide binding, the binding of sulphide is apparently independent of the redox state of components of the oxidase other than cytochrome a_3 and shows no anomalous kinetics during complex formation.

3. Sulphide binding to cytochrome a_3^{3+} is accompanied by a blue-shift in the α-peak of the reduced enzyme ($a^{2+}\ a_3^{3+}\ H_2S$), similar to but smaller than that induced by azide.

4. The reduced sulphide-inhibited system shows a much higher Soret peak at 445 nm than the corresponding cyanide and azide complexes, suggesting that partial electron transfer from sulphide to haem may occur in the complex. No evidence was obtained for the formation of any sulfhaem derivatives of cytochrome a_3.

5. The influence of energization on the spectrum of mitochondrial cytochrome oxidase, and the effects of calcium on the α-peak of isolated cytochrome aa_3 (Wikström, M. K. F. (1974) Ann. N. Y. Acad. Sci. 227, 146–158) are distinct from the action of the cytochrome a_3 ligands.

6. A classification of peak shifts in the α-region in terms of isosteric and allosteric ligands is proposed.

INTRODUCTION

Cyanide was the classical terminal inhibitor of respiratory chain electron transfer [1] and was not surprisingly used in the initial spectrophotometric studies of such systems [2]. Later on, azide, introduced as an inhibitor by Keilin [3], was employed in preference to cyanide [4]. Finally observations were made with sulphide as the terminal inhibitor rather than either azide or cyanide [5].

The use of cyanide as a routine inhibitor was abandoned because of its slow and complex pattern of binding. An attempt to analyse the kinetics of cyanide binding has been made previously [6, 7]. The use of azide was perhaps abandoned because,

although it reacts rapidly and simply [8], when complexed to the enzyme it induces a blue shift of the α-band of reduced cytochrome *a*, presumably through some form of haem-haem interaction [9, 10]. The kinetics and spectroscopic consequences of sulphide inhibition have not been analysed previously, however. The present paper presents the beginning of such an analysis.

In the course of this study it was found that a small blue shift in the cytochrome aa_3 α-peak is induced by sulphide. A comparative study was therefore undertaken of the two types of α-peak shift that have been reported for cytochrome *c* oxidase. Blue shifts seem to be characteristic of ligands binding to the a_3 haem (azide, sulphide, and possibly carbon monoxide), while red shifts are characteristic of conditions or ligands of the 'allosteric' type, such as the energization effect [11–13] and the action of calcium recently reported by Wikström [14].

MATERIALS AND METHODS

Keilin-Hartree type beef heart submitochondrial particles were prepared essentially as described previously [8]. Maximal activity towards cytochrome *c* oxidation was induced by treating particles (10 mg/ml medium A below) with 1 % deoxycholate before assay, as recommended by Smith and Camerino [15]. Cytochrome *c* oxidase (cytochrome aa_3) was prepared from such particles according to van Buuren [16] (Fowler-type preparation) or according to Yonetani [17] (Yonetani-type preparation). Spectra were obtained with a Cary 118c spectrophotometer. Kinetic studies under steady state conditions were carried out with the same instrument or with a Perkin-Elmer/Hitachi 356 dual wavelength spectrophotometer. Oxygen uptake was monitored with a Radiometer O_2 electrode coupled to an appropriate amplifier and recorder; reaction volume was 3.8 ml and temperature 30 °C for polarographic experiments.

Sodium sulphide was prepared from the solid kept at 4 °C (Merck Analytical reagent). Its concentration was checked by titration of metmyoglobin (Sigma sperm whale) at pH 7.4 with freshly made solutions ($K_d \approx 20\,\mu M$) and found to correlate closely with the estimated concentration by weight ($\geqslant 90\,\%$). Cytochrome *c* was Sigma Type VI (horse heart). Other reagents and methods were of the usual laboratory kind. Medium 'A' for storage or sodium deoxycholate treatment of Keilin-Hartree particles contains: 0.125 M Na_2HPO_4, 0.0625 M boric acid, 0.01 M Na_2-EDTA, 20 % w/v sucrose, adjusted to pH 7.5.

RESULTS

Fig. 1 compares the spectra of the three inhibited forms of cytochrome aa_3 reduced with ascorbate plus *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD). Fig. 1A shows the sulphide-inhibited form ($a^{2+}\ a_3{}^{3+}\ H_2S$) and Figs 1B and 1C the cyanide- and azide-inhibited forms ($a^{2+}\ a_3{}^{3+}$ HCN and $a^{2+}\ a_3{}^{3+}\ HN_3$) obtained for the Fowler-type enzyme [16] under the same conditions, for comparison. Although the change in the position of the α-peak is less marked than in the case of azide (1C), there is little doubt that a blue shift occurs in the case of sulphide (1A) that does not take place with cyanide (1B). Observations with several different preparations of cytochrome aa_3 have confirmed this, and show that the peak shift is about 1 nm towards the blue.

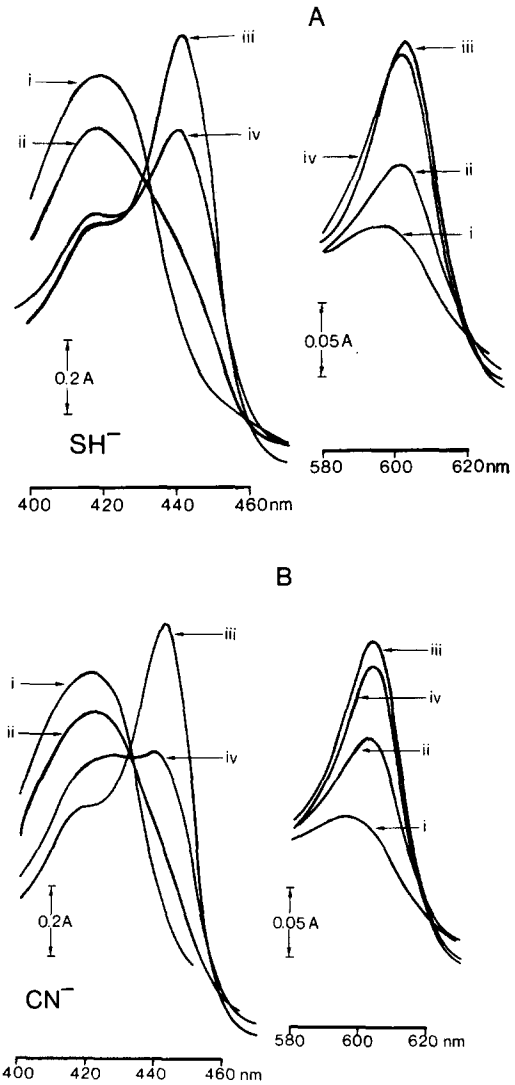


Fig. 1. See opposite page for legend.

The Soret bands of the various inhibited oxidases also show an unusual feature in the case of sulphide (cf. Fig. 1A with Figs 1B and 1C), namely that the 445-nm shoulder, usually attributed to ferrous cytochrome *a*, is much higher in the a^{2+} $a_3{}^{3+}$ H_2S system than with the other inhibitors. The low temperature spectra of Gilmour et al. [18] show a similar effect at 440 nm, although these workers did not comment on the result, nor report any blue shift in the α-peak region resembling that shown in Fig. 1A.

The spectral difference at 445 nm can be used to distinguish between the formation of the sulphide- and cyanide-inhibited species. Fig. 2 illustrates the differences observed, and shows that cyanide (from 67 μM to 2 mM) was incapable of

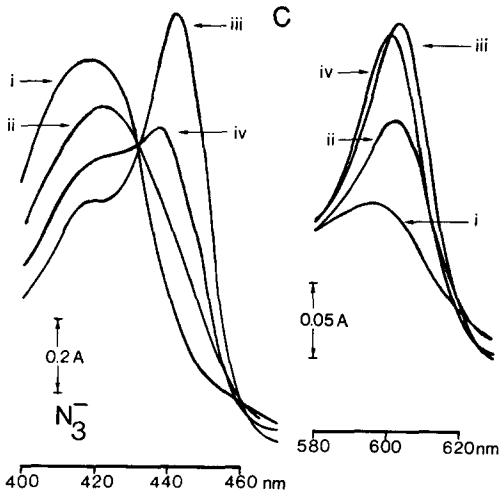


Fig. 1. Spectral changes induced by sulphide, cyanide and azide. 5.5 μM cytochrome aa_3 (Fowler-type [16]) in 67 mM phosphate pH 7.4, 0.5 % Tween-80, in (i) oxidized state, (ii) aerobic steady state in presence of 17 mM ascorbate plus 330 μM TMPD, (iii) fully reduced (anaerobic) state, and (iv) inhibited steady state in presence of inhibitor: (A) 2 mM sodium sulphide; (B) 67 μM potassium cyanide; (C) 3.3 mM sodium azide.

displacing sulphide (2 mM) rapidly from the haem. Similarly addition of sulphide to the preformed cyanide complex had no effect on the cyanide inhibited spectrum over periods of up to 1 h.

A study of the reaction of sulphide with fully oxidized enzyme (a^{3+} $a_3{}^{3+}$),

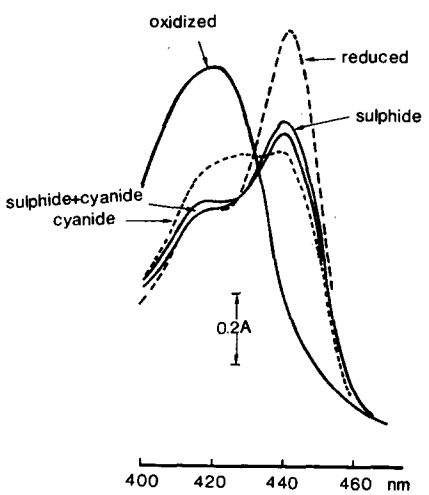


Fig. 2. Absence of cyanide effect on the Soret peak of cytochrome aa_3 in the presence of reductant and sulphide. 5.5 μM cytochrome aa_3 (Fowler-type [16]) in 67 mM phosphate pH 7.4, 0.5 % Tween-80. (a) ———, oxidized; (b) – – –, reduced (anaerobic) in presence of 17 mM ascorbate plus 330 μM TMPD; (c) - - -, reduced (inhibited steady state) in presence of ascorbate, TMPD and 67 μM cyanide; (d) —, reduced (inhibited steady state) in presence of ascorbate, TMPD and 2 mM sulphide; (e) —, sulphide-inhibited spectrum (d) after addition of 2 mM cyanide (10 min equilibration period).

which has been carried out for the inhibitors cyanide [6], azide [19] and fluoride [20], was not possible. Sulphide is capable of slowly reducing the oxidized enzyme; but it is destroyed by ferricyanide, the usual additive to keep the oxidase ferric. The oxidized enzyme and submitochondrial particles also react with low levels of sulphide to remove the latter from the system, perhaps by addition to dithiobridges and elsewhere.

However, in a catalytically active system containing ascorbate, cytochrome *c*, cytochrome aa_3 and O_2, addition of sulphide induces a progressive inhibition of cytochrome *c* oxidase, that can be monitored by the increase in steady state reduction of cytochrome *c*. Sulphide, like cyanide [7], reacts relatively slowly with the enzyme. But when such steady state data are analysed in terms of the actual decline in catalytic activity (reactive aa_3 units) according to the equation [7]:

$$\frac{[aa_3]_t}{[aa_3]_o} = \frac{[c_{ox}]_t}{[c_{red}]_t} \cdot \frac{[c_{red}]_o}{[c_{ox}]_o} \qquad (1)$$

then results are obtained as plotted in Fig. 3. Unlike cyanide, the sulphide binding is monophasic, with a rate constant rather greater than $10^4\ M^{-1}.\ s^{-1}$.

Fig. 2 above showed that cyanide could not readily replace bound sulphide. Fig. 4 shows the rate of appearance of the full 445 nm peak on the addition of dithionite to the sulphide inhibited enzyme. A half time of some 20 min was observed under these conditions. Table I lists the K_i values for sulphide inhibition obtained directly from steady state experiments, together with the rather lower estimates from the rates of the 'on' and 'off' reactions. In addition values obtained polarographically for the inhibition of ascorbate-TMPD oxidation by cytochrome aa_3 and for inhibition of succinate oxidation by Keilin-Hartree submitochondrial particles

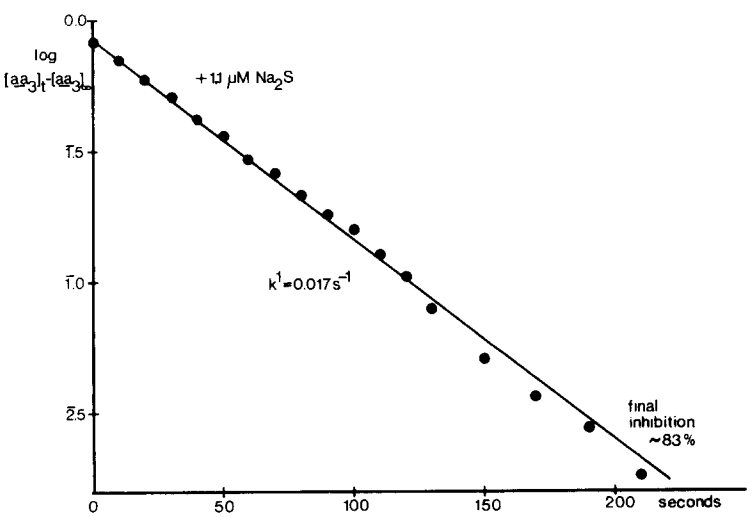


Fig. 3. Kinetics of cytochrome *c* oxidase inhibition by sulphide. 67 mM potassium phosphate, pH 7.4, plus 0.5 % Tween-80, 30 μM horse heart cytochrome *c*, 0.75 mM ascorbate plus 93 nM cytochrome aa_3 (in the form of deoxycholate treated submitochondrial particles [15], used to ensure maximal turnover in the uninhibited system). Initial steady state reduction 32 %, changes observed following addition of 1.1 μM Na_2S. Final steady state reduction 73 %, corresponding to 83 % inhibition with an apparent K_i of 0.24 μM. Enzyme concentration calculated according to Eqn (1) [7].

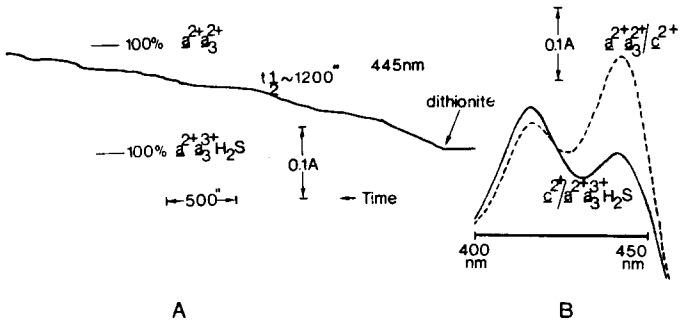


Fig. 4. Reduction of cytochrome a^{2+} $a_3{}^{3+}$ H_2S complex by dithionite. Rate of H_2S dissociation. 5 mM potassium phosphate, pH 7.8, plus 0.5 % Tween-20 (low phosphate medium secures maximum cytochrome *c* binding). 1.7 μM cytochrome aa_3 (Yonetani-type) [17], 1.25 μM horse heart cytochrome *c*, 5.6 mM ascorbate, 380 μM TMPD, in presence and absence of 15 μM Na_2S. B (right hand side). Soret region spectra in anaerobic state (- - -) and sulphide-inhibited state (—). A (left hand side). Kinetics of H_2S dissociation followed at 445 nm by slow reduction induced by addition of dithionite.

are listed. The slightly higher direct values probably reflect sulphide losses as discussed above. However, it is clear that the binding of sulphide to the catalytically active enzyme is approximately the same as that of cyanide [6, 7], and much tighter than the binding of ligands like azide and fluoride.

The blue shift of the α-peak induced by sulphide, together with the recognized blue shift, produced by azide [9, 10], raised the question as to whether all such ligands tend to induce blue shifts. Wikström [14] has reported a red shift of this peak that occurs in mitochondria in the presence of calcium. Wikström and Saari (personal communication) have demonstrated a similar red shift on addition of calcium salts

TABLE I

INHIBITION CONSTANTS FOR SULPHIDE AND CYTOCHROME *c* OXIDASES OR SUBMITOCHONDRIAL PARTICLES

Conditions	Substrate	k_{on} $(M^{-1} \cdot s^{-1})$	k_{off} (s^{-1})	k_{off}/k_{on} (μM)	K_i (μM)
1. Cytochrome aa_3:[a]					
Cytochrome *c* steady state[b]	ascorbate	$1.5 \cdot 10^4$	$6 \cdot 10^{-4}$[d]	0.04	0.24
Polarography[c]	ascorbate + TMPD	$1.2 \cdot 10^4$	$\leq 2 \cdot 10^{-3}$[e]	≤ 0.2	0.3
2. Keilin-Hartree submitochondrial particles:					
Polarography[f]	Succinate	$0.8 \cdot 10^4$	$\leq 10^{-3}$[e]	≤ 0.12	1.0

[a] Yonetani-type preparation (see Methods).

[b] 67 mM phosphate, pH 7.4, 0.5 % Tween-80, 0.7 mM ascorbate, 30 μM cytochrome *c*, 0.1 μM cyt. aa_3, measurements at 550 nm (cf. Fig. 3), 27 °C.

[c] 67 mM phosphate pH 7.4, 0.5 % Tween-80, 30 μM cytochrome *c*, 7.9 mM ascorbate, 0.26 mM TMPD, 0.03 or 0.06 μM cyt. aa_3, 30 °C.

[d] From experiment of Fig. 4 (5 mM phosphate, pH 7.8).

[e] Maximal estimate from inhibition time course.

[f] 67 mM phosphate, pH 7.4, 0.5 % Tween-80, 3 μM cytochrome *c*, 10 mM succinate, approx. 0.08 μM aa_3 as submitochondrial particles, 30 °C.

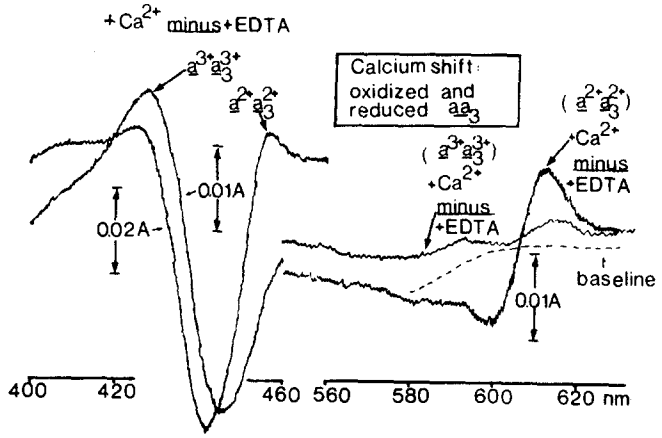


Fig. 5. Calcium-induced difference spectra with oxidized and reduced cytochrome aa_3. 5.5 μM cytochrome aa_3 (Fowler-type) (in both cuvettes) in 67 mM phosphate pH 7.4, 0.5 % Tween-80. 80 μM $CaCl_2$ added to sample cuvette, and 240 μM EDTA to reference cuvette (to remove residual Ca^{2+} in buffer system [14]). Reduction with slight excess of dithionite.

to isolated cytochrome aa_3 of the Yonetani-type [17]. Fig. 5 illustrates the calcium shift obtained with isolated cytochrome aa_3 of the Fowler type, both in the fully oxidized state, where very little effect is seen in the visible region, and in the fully

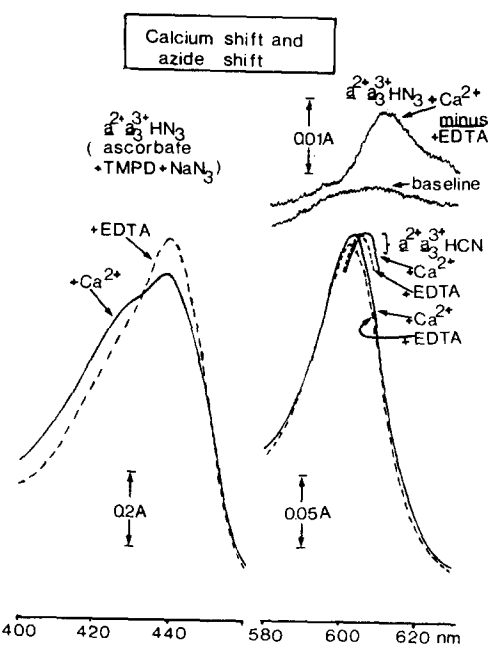


Fig. 6. Calcium shifts in aerobic azide-inhibited steady state: absolute and difference spectra. 5.5 μM cytochrome aa_3 (Fowler-type) in 67 mM phosphate pH 7.4, 0.5 % Tween-80. Spectra in presence of 12 mM ascorbate, 200 μM TMPD plus 10 mM NaN_3 as indicated. Corresponding cyanide-inhibited spectra added in 600–610 nm region for comparison. —, plus 80 μM $CaCl_2$; - - -, plus 240 μM EDTA. Inset: Difference spectrum for azide-inhibited steady state ($+Ca^{2+}$ minus +EDTA).

reduced state (Ca^{2+} in sample, EDTA in reference cuvette), where a red shift is seen at 605 nm together with a marked bathochromicity at 445 nm.

According to Wikström and Saari (personal communication) the calcium shift can be seen equally well in the cyanide-inhibited reduced state, indicating that it is not abolished by ligand binding to cytochrome $a_3{}^{3+}$ haem, and that, according to classical criteria, it is therefore attributable to a change in cytochrome a^{2+}, in the same way as the azide- and sulphide-induced blue shifts. Fig. 6 illustrates the result obtained in the present system when azide and calcium are present simultaneously. The azide-induced blue shift is still seen in the presence of Ca^{2+}; but the Ca^{2+}-induced red shift is diminished somewhat in the presence of azide.

DISCUSSION

Table II lists the K_i values for various cytochrome a_3 haem ligands, obtained experimentally in terms of total ligand, and also calculated in terms of undissociated acid concentration. Sulphide resembles cyanide in the slowness of the 'off' reaction and the consequent tightness of binding, while azide and (probably) fluoride are more readily reversible inhibitors. It is possible that sulphide also behaves like cyanide [7] in reacting more slowly with the fully oxidized than with the partially reduced enzyme, because sulphide added before substrate tended to 'disappear', while that added during the steady state was more effective. On the other hand, it may be that the secondary sites for sulphide binding are more readily accessible in the fully oxidized state. Sulphide resembles azide in its relatively simple binding kinetics and its induction of a blue shift in the 605 nm peak. Table III summarises the shifts in the α-peak of cytochrome aa_3 that have been reported to date, both in the soluble enzyme and in the mitochondrion. In general, haem a_3 ligands giving low spin complexes are red-shifted compared to ligands expected to form high spin complexes.

TABLE II

DISSOCIATION CONSTANTS FOR COMPLEXES OF CYTOCHROME $a_3{}^{3+}$ WITH FOUR LIGANDS

All data for pH 7.0–8.0, 25–30 °C.

Ligand	apparent K_i (μM)	k_{off} (s^{-1})	pK	K_i'* (μM)	Ref.
H_2S	0.04–0.20	$\approx 10^{-3}$	7.0	≈ 0.02	this paper
HCN	0.07–0.15	$\approx 2 \cdot 10^{-3}$	9.3	0.07	7
HN_3	50–80	0.4	4.7	0.1	19
HF	10 000	?	3.5	2.0	20

* Calculated in terms of the undissociated acid present (in the case of fluoride [20], the binding species remains uncertain).

Sulphide is unique among the a_3 ligands in giving rise to an appreciable 445 nm peak in the a^{2+} $a_3{}^{3+}$ H_2S complex (Figs 1 and 2). Two interpretations are possible:

TABLE III

SHIFTS OF THE α-PEAK IN CYTOCHROME aa_3 UNDER DIFFERENT LIGANDING AND REDOX CONDITIONS

All data refer to room temperature (25–30 °C) and approximately neutral pH (6.5–7.5).

Enzyme form	Absolute α-maximum (nm)	Reference
(a) "Normal" (605 nm) spectra:		
1. $a^{2+}\ a_3{}^{2+}$ (anaerobic)	605	Keilin [25], Lemberg [23]
2. $a^{2+}\ a_3{}^{3+}$ HCN	605	Nicholls [22]
3. $a^{2+}\ a_3{}^{3+}$ (steady state)[a]	605	Nicholls and Kimelberg [10]
4. $a^{2+}\ a_3{}^{3+}$ (stopped flow-early reduction)[b]	605	Andréasson [26]
(b) Blue-shifted spectra:		
5. $a^{2+}\ a_3{}^{3+}$ HN_3	602.5	Wilson and Chance [9]
6. $a^{2+}\ a_3{}^{3+}$ H_2S	604	this paper
7. $a^{2+}\ a_3{}^{2+}$ CO (?)[c]	604(?)	Vanneste [27]
8. $a^{2+}\ a_3{}^{3+}/a^{3+}\ a_3{}^{2+}$ (anaerobic reduction)[d]	603.5	Tiesjema et al. [24]
9. $a^{2+}\ a_3{}^{3+}$ (reoxidized with ATP+malate)[e]	603.5	Muraoka and Sugiyama [28]
10. $a^{2+}\ a_3{}^{3+}$ (stopped flow-final reduction)[b]	603.5	Andréasson [26]
(c) Red-shifted spectra:[f]		
11. $a^{2+}\ a_3{}^{2+}$ (+ATP in situ)	606.5	Wikström [13]
12. $a^{2+}\ a_3{}^{2+}$ CO (+ATP in situ)[g]	606.5	Lindsay [29]
13. $a^{2+}\ a_3{}^{2+}$ ($+Ca^{2+}$)	606.5	Wikström [14]
14. $a^{2+}\ a_3{}^{3+}$ HCN ($+Ca^{2+}$)[h]	606.0	Wikström and Saari (personal communication)

[a] In the aerobic steady state, a_3 may be in the low spin ("oxygenated") rather than a high spin ferric state.

[b] On mixing ferrocytochrome *c* with ferric cyt. aa_3 an initial reduced form at 605 nm changes, without further reduction, to a final reduced form with α-peak at 603.5 nm.

[c] The shift in the cyt. *a* α-peak is obscured by the appearance of the $a_3{}^{2+}$ CO peak.

[d] The α-peak of the half-reduced state is assumed here to be largely due to cyt. *a*.

[e] Others [11, 12, 29] have found that the initial reduced peak in energized mitochondria is blue-shifted.

[f] Excluding red-shifted spectra obtained by subtracting a blue-shifted from a normal spectrum-an artificial procedure (cf. refs [21, 24]).

[g] The ATP-shift has also been reported, but not shown, for the $a^{2+}\ a_3{}^{3+}$ HCN complex.

[h] But Ca^{2+} does not overcome completely the blue-shift induced by azide (this paper).

(a) an 'allosteric' effect of $a_3{}^{3+}$ H_2S on the Soret absorption coefficient of cytochrome *a*, similar to the effect on the α-peak postulated by Wilson et al. [21], and

(b) a partial reduction of the a_3 haem in its complex with H_2S, according to Eqn 2:

$$\underline{a_3^{3+}SH^- \cdots H^+Y} \leftrightarrow \underline{a_3^{2+}SH^{\cdot} \cdots H^+Y} \qquad (2)$$

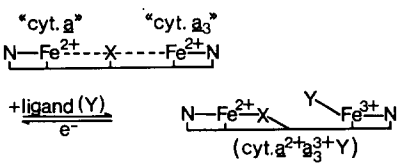
However caused, it facilitates the direct observation of sulphide binding as compared to azide or cyanide binding (Fig. 2). Clearly, both sulphide and cyanide bind tightly and it is difficult to exchange one ligand for the other. And the spectral differences confirm that the three ligands bind either to the same site (cytochrome a_3^{3+}) or to sites with strongly anticooperative interactions.

Nicholls and Kimelberg [10] raised the question as to whether there was an appreciable contribution of the a_3^{2+} haem to the α-peak of the fully reduced enzyme. The present author has pursued this point in a comparison of the action of cyanide and azide on the purified oxidase of the Fowler type [22]. Fig. 1 reemphasizes this for the case of the sulphide-inhibited enzyme. There is no indication of a specific difference spectrum in the 580–625 nm region that might be characteristic of 'a_3^{2+} minus a_3^{3+} H_2S'. Two interpretations are possible again:

(a) that due to Lemberg [23], proposing a high-spin reduced haemoglobin structure for cytochrome a_3^{2+}, with a consequent spectrum rather similar to the spectra of low-spin ferric complexes; and

(b) that due to Wilson and coworkers [21], proposing a haem-haem interaction between liganded cytochromes a_3^{3+} and a^{2+}, increasing the difference extinction coefficient of the latter almost twofold at 605 nm.

The present author still prefers interpretation (a), as the coincidence of values required by interpretation (b) is hard to understand, unless one postulates a rather rigid interaction model with initially indistinguishable, or almost indistinguishable haems [24], as in Eqn 3:



(3)

The construction of Table III enables certain predictions to be made about the behaviour of the reduced α-peak under different conditions, established either by different redox or ligand states of the other haem group (cytochrome a_3) or by changes of an indirect (allosteric) kind including the effect of Ca^{2+} and of membrane energization in the intact mitochondrion [11–14].

Ligands acting at the haem iron of cytochrome a_3 either produce no change (HCN) or a blue shift (HN_3, H_2S, CO?) in the ferrocytochrome *a* spectrum. This shift resembles that seen in the partially reduced anaerobic state, in free enzyme (Table III, 8) and in mitochondria (Table III, 9). Indirect effects shift the cytochrome *a* α-peak in the opposite direction; in this respect Ca^{2+} binding mimics mitochondrial energization [14].

The system seen here is reminiscent of the effects on the spectrum of haemoglobin induced by haem ligands such as CO and HCN, and by allosteric ligands such as inositol hexaphosphate [30]. It may however be some time before the corresponding structural information is available in the case of cytochrome aa_3. Meanwhile the scheme of Fig. 7 can be put forward in an attempt to summarize the observations.

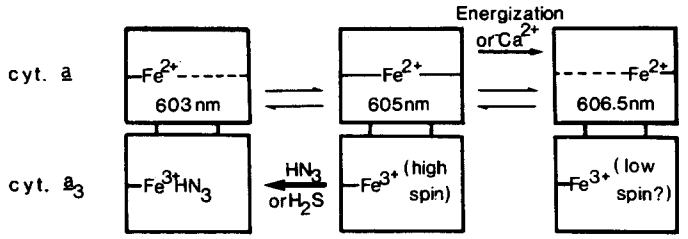


Fig. 7. Scheme for ligand-induced and allosterically-induced α-peak shifts in cytochrome aa_3.

The reduced cytochrome *a* α-peak can lie at 606.5, 605 or 603 nm. Shifts towards the blue form are induced by azide and sulphide binding to the ferric a_3 haem. Shift towards the red form are induced by Ca^{2+} or by mitochondrial energization. The latter also induces a high → low spin shift in the spectrum of ferric a_3 haem in the fully oxidized system [31]. Whether this spin state shift is related to the spectral shifts of the other haem (cytochrome *a*), as might be expected from the haemoglobin analogy [30], remains to be studied.

ACKNOWLEDGEMENTS

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