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Review

The mixed valence state of the oxidase binuclear centre: how *Thermus thermophilus* cytochrome ba_3 differs from classical aa_3 in the aerobic steady state and when inhibited by cyanide

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Abstract

In the aerobic steady state of the classical eukaryotic cytochrome *c* oxidase, three aa_3 redox metal centres (cytochrome *a*, CuA and CuB) are partially reduced while the fourth, cytochrome a_3, remains almost fully oxidized. Turnover depends primarily upon the rate of cytochrome a_3 reduction. When prokaryotic cytochrome *c*-552 oxidase (ba_3) of *Thermus thermophilus* turns over, three different metal centres (cytochromes *b*, a_3 and CuA) share the steady state electrons; it is the fourth, CuB, that apparently remains almost fully oxidized until anaerobiosis. Cytochrome a_3 stays partially reduced during turnover and a possible P/F state may also be populated. Cyanide traps the aerobic ba_3 CuB centre in the a_3^{2+}CNCuB^{2+} state; the corresponding eukaryotic cyanide trapped state is a_3^{3+}CNCuB^{+}. Both states become the fully reduced a_3^{2+}CNCuB^{+} upon anaerobiosis.

The different reactivities of the aa_3 and ba_3 binuclear centres may be correlated with the very different proximal histidine εN-Fe distances in the two enzymes (3.3 Å for ba_3 compared to 1.9 Å for aa_3) which may in turn relate to the functioning of thermophilic *Thermus* cytochrome ba_3 in vivo at a very elevated temperature. But the differences may also just exemplify how evolution can find surprisingly different solutions to the common problem of electron transfer to oxygen. Some of these alternatives were potentially enshrined in a model of the oxidase reaction already adopted by Gerry Babcock in the early 1990s.

1. Introduction

In 1992 Gerald Babcock together with Mårten Wikström published what may have been the last major review of cytochrome *c* oxidase mechanisms [1] prior to the structural determinations for both bacterial [2] and mammalian [3,4] enzymes. In this review, they formalised the now accepted sequence of reaction intermediates involved in reduction of oxygen at the binuclear Fea_3-CuB centre of the classical cytochrome oxidases (cf. Fig. 1). Much of this model has withstood the test of time and the detailed knowledge of the molecular structure available to us in the decade that followed.

But in one sense the model was especially prescient. It may be noted that the ''electronated'' [5] species numbered 11 in the cycle is designated as a binuclear centre with effective charge 4+, which could equally well represent ferric iron and cuprous copper or ferrous iron and cupric copper. In a critique of this model [6], the present senior author had no such inhibitions. The electronated (one-electron) state was firmly designated as comprising ferric heme iron and cuprous CuB, partly to fit the alternative model being proposed and partly in consequence of the experimental evidence available for the state of the mammalian enzyme during aerobic turnover. No reduction of the heme a_3 can be seen in the aerobic steady state until very low levels of oxygen are reached, but a high spin iron electron paramagnetic resonance (epr) signal is present, presumably characteristic of the breakage in the antiferromagnetic coupling between Fe and Cu as a result of CuB reduction.

Cyanide is the classic inhibitor of cell respiration and cytochrome oxidase activity [7,8] and also a probe of the

Abbreviations: epr, electron paramagnetic resonance; TMPD, *N*,*N*,*N*′,*N*′ tetramethyl-*p*-phenylene diamine; CN, cyanide

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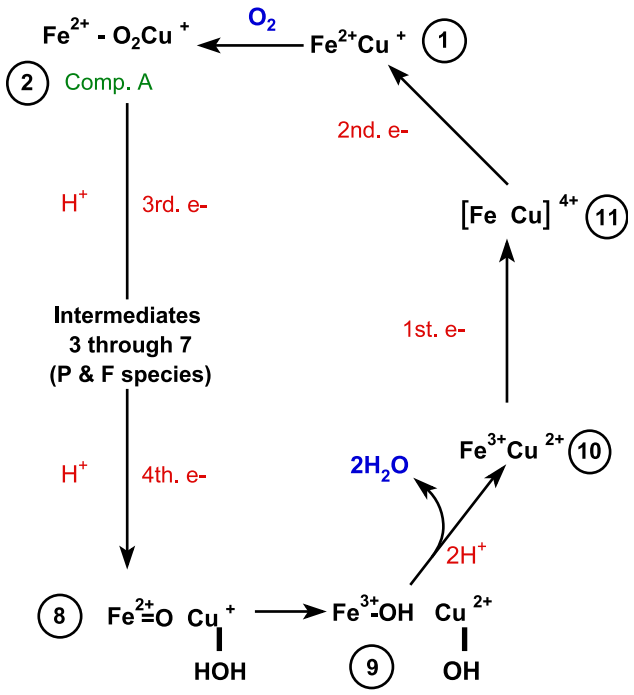


Fig. 1. The Babcock–Wikström 1992 cyclic model for cytochrome oxidase turnover. Redrawn from Ref. [1]. A major focus of the original article was upon the events following the oxygen reaction (the intermediates 3 through 7 are omitted here). Current thinking may also see some protonations taking place during the reductive phase (intermediates 10–11–1 rather than 9–10).

catalytic mechanism and of models such as that in Fig. 1. The kinetics of cyanide inhibition of the enzyme are complex [9,10] but most simply interpreted in terms of a very slowly reversible binding of cyanide to the fully oxidized enzyme, a rapid and tight binding to the mixed valence state intermediate, and a much weaker and fully reversible binding to the fully reduced state [11,12]. The predicted mixed valence intermediate, which is also the species primarily responsible for inhibition, then has the structure $a_3Fe^{3+}HCNCuB^{+}$ and shows the expected epr signal characteristic of a low spin ferric iron ligated state, in this case with a g_x band at 3.58 [13,14]. This species represents the cyanide trapped form of the "electronated" intermediate 11 in the Babcock–Wikström cycle.

So the consensus remained until other oxidases began to be examined in the last two decades of the 20th century. One of these was the terminal oxidase of the thermophilic bacterium *Thermus thermophilus*, cytochrome ba_3 [15,16]. This enzyme contains the same five metal centres seen in classical cytochrome oxidase, including the binuclear CuA centre and a CuB coupled to heme iron at the binuclear oxygen-reactive centre, but with a protoheme replacing cytochrome *a* and a slightly modified a_3 heme in which a geranyl–geranyl group replaces the farnesyl side chain of the classical a_3, thus providing the basis for a modified nomenclature of cytochrome a_3 [15]. Early in the study of this evolutionary analogue of the classical cytochrome aa_3, it became obvious that there were substantial functional differences. Cyanide binding studies suggested that the stable cyanide-inhibited form contained ferrous cytochrome a_3 rather than cuprous CuB [17]. Oertling et al. [18] were subsequently able to prepare and define by a combination of techniques cyanide complexes at several enzyme reduction levels, and showed that unlike the classical enzyme, at least two of the redox species involved, one-electron and three-electron reduced, had cupric CuB with epr spectra showing typical nitrogen hyperfine structure.

The relationship of these intermediates to the inhibited forms of the enzyme under catalytic conditions remained problematic, as in the case of the corresponding classical cyanide compounds [12]. The latter question was taken up subsequently both by Siletskiy et al. [19] and by Giuffrè et al. [20], the former in a study of vesicle-reconstituted enzyme and the latter in a kinetic and temperature study of the isolated enzyme. Both groups were able to show that although ba_3 is much slower than aa_3 to react with cyanide to form an inhibited species under turnover conditions, effective cyanide binding can be achieved following electron injection, as with aa_3.

We therefore thought it worthwhile to look again at the ba_3 steady state, both in the presence and absence of cyanide as terminal inhibitor, to compare with previous studies of the classical cytochrome aa_3 and thus if possible to devise an overall model for the oxidase catalytic activity that would encompass both types of enzyme. The present paper represents a modest component of an ongoing programme.

2. Methods and materials

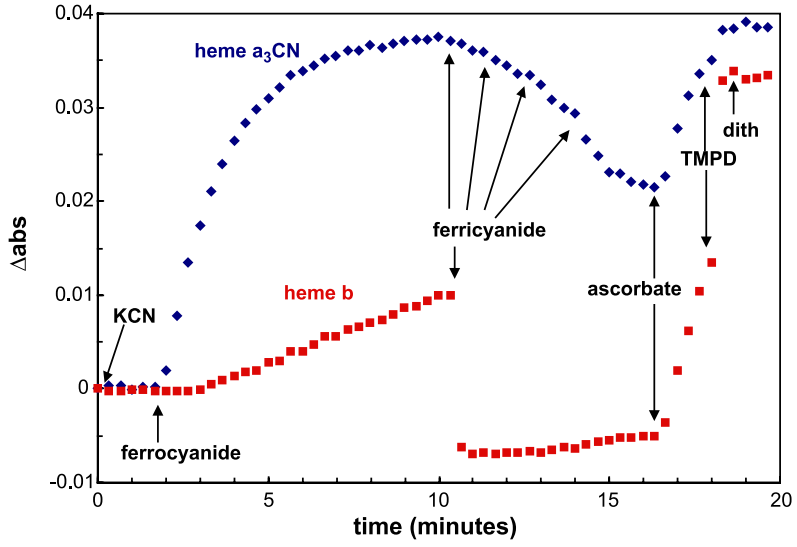
2.1. Enzyme preparation

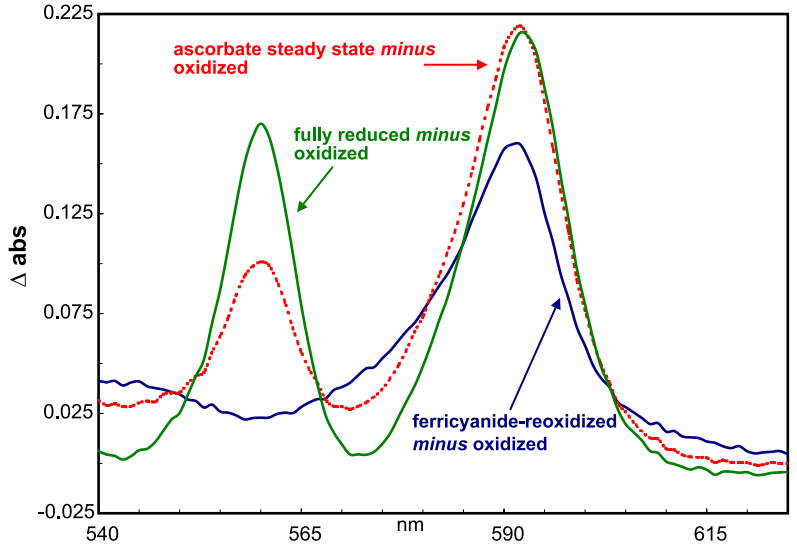
Cytochrome ba_3 from *T. thermophilus* was prepared as previously described [21], and samples were stored at −80 °C in potassium phosphate lauryl maltoside buffer pH 7.

2.2. Materials

N,*N*,*N*′,*N*′ tetramethyl-*p*-phenylene diamine (dihydrochloride; TMPD) and ascorbic acid (sodium salt) were Sigma products. Buffer salts, potassium ferri- and ferrocyanide, sodium and potassium cyanide, and all other reagents were of AnalaR™ or similar quality.

Fig. 2. Formation of the *T. thermophilus* cytochrome ba_3 cyanide complex. (A) Time course of cyanide complex formation: effect of ferrocyanide and ferricyanide on the reaction, 2 μM cytochrome ba_3 at pH 7.55 in 75 mM potassium phosphate buffer 31 °C. Additions of 5 mM KCN and 3.9 mM $K_4Fe(CN)_6$ as indicated, followed by successive additions amounting to 39, 78, 235 and 530 μM $K_3Fe(CN)_6$. Final reduction with 10 mM ascorbate, 0.19 mM TMPD, and dithionite. Heme *b* reduction monitored at 560–574 nm; cyanide a_3 reduction monitored at 590–574 nm. (B) Difference spectra (visible region) of the intermediates in ba_3 cyanide complex formation: conditions as in panel A. (C) Redox equilibrium between the oxidized and reduced forms of the ba_3 cyanide complex: conditions as in panel A. Measurements at pH 7.5, 30 °C, assuming E_0' (ferri/ferrocyanide) = +420 mV and RT/$\mathscr{F}$ = 59 mV/decade.


heme a3CN
heme b
ferricyanide
ascorbate
TMPD
dith
KCN
ferrocyanide
Δabs
time (minutes)


ascorbate steady state minus oxidized
fully reduced minus oxidized
ferricyanide-reoxidized minus oxidized
Δ abs
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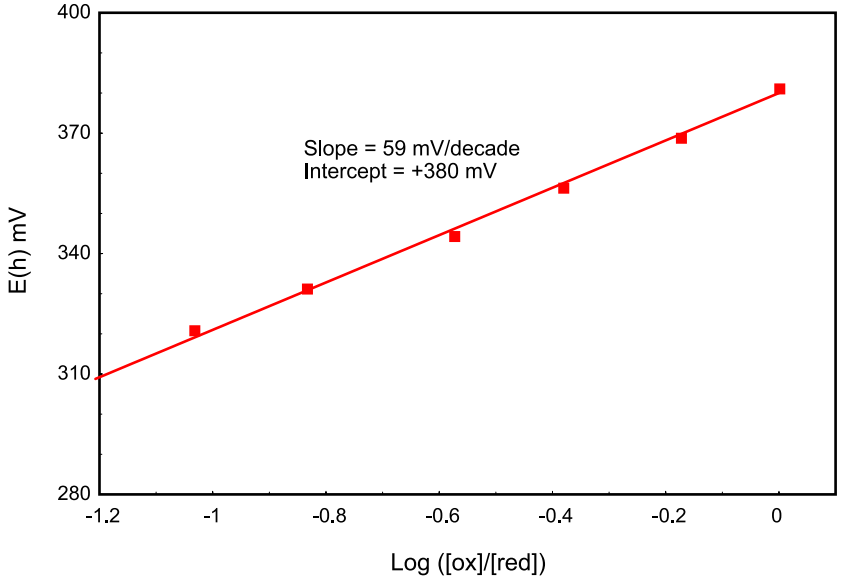
Slope = 59 mV/decade
Intercept = +380 mV
E(h) mV
Log ([ox]/[red])

2.3. Steady state spectrophotometry

Steady state spectrophotometry was carried out with a Hewlett-Packard diode array spectrophotometer HP8543 linked to a computer. Data analysis was carried out either with the resident HPChem software or by exporting the data as Microsoft Excel files.

Extinction coefficients determined or employed (cf. Refs. [17,18]) were:

ΔE_{mM} (613–658 nm), reduced *minus* oxidized, for heme a_3=6.3
ΔE_{mM} (560–574 nm), reduced *minus* oxidized, for heme b_3=19.5.

3. Results and discussion

Fig. 2 shows the formation of the ba_3-cyanide complex as well as the reduction of the heme *b* group by ferrocyanide in the presence of KCN. The time courses of ba_3-CN and ferrocytochrome *b* appearance are illustrated in Fig. 2A. Addition of cyanide to the fully oxidized enzyme has little spectroscopic effect; but the addition of the very weak reductant ferrocyanide induces an immediate appearance of the characteristic spectrum of a_3CN, followed after a short lag phase by the reduction of heme *b*. Subsequent addition of ferricyanide aliquots to poise the system completely reoxidize the *b* heme and progressively reoxidize the a_3CN. Rereduction to completeness can be achieved by addition

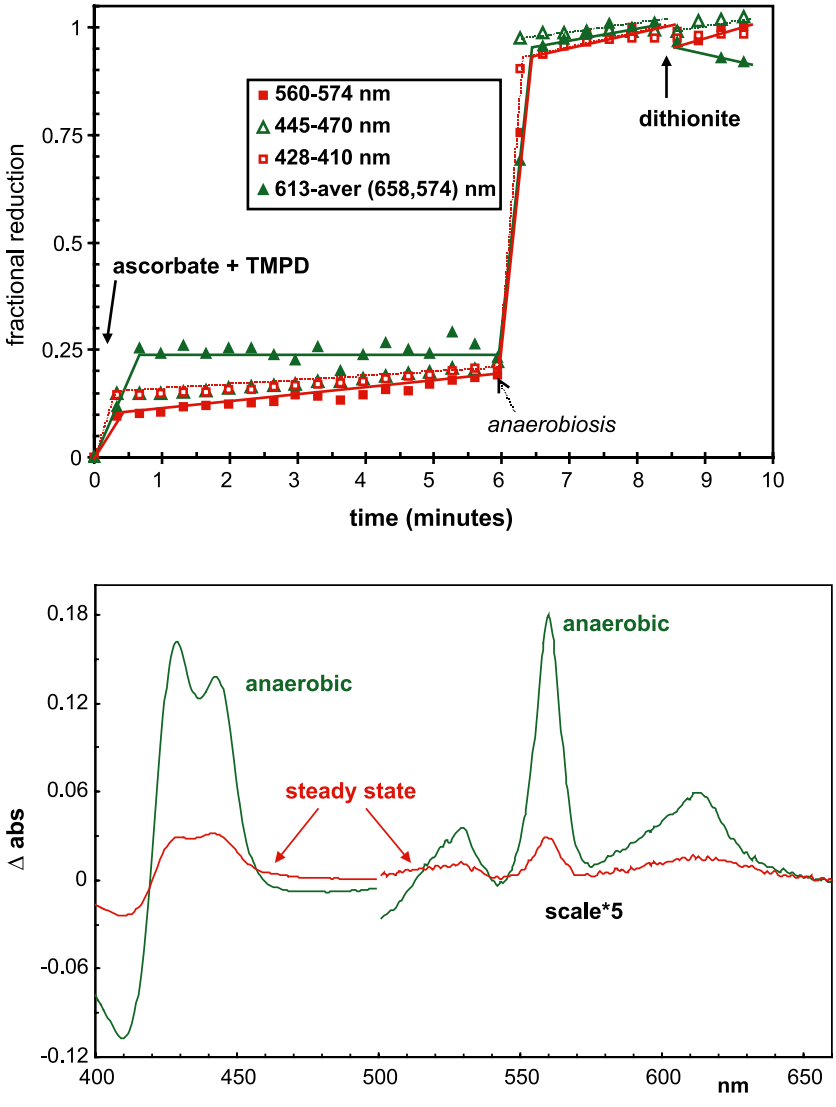

Fig. 3. Aerobic steady state of cytochrome ba_3 oxidizing ascorbate plus TMPD. (A) Time courses of reduction of the *b* and a_3 hemes: 2 μM cytochrome ba_3 at pH 6.9 in 80 mM aerobic potassium phosphate buffer at 29 °C. Additions of 0.2 mM TMPD, 10 mM potassium ascorbate and dithionite as indicated. Fractional reductions monitored at 560 *minus* 574 nm (visible, lower closed squares) and 428–410 nm (Soret, open squares) for heme *b* and 613 *minus* average (658, 574 nm) (visible, upper closed triangles) and 445–470 nm (Soret, open triangles) for heme a_3. (B) Difference spectra of the enzyme in steady state and after anaerobiosis. Conditions as in panel A; spectra immediately before and after anaerobiosis; visible region scale expanded 5-fold; visible region spectrum corrected for spectral contribution due to residual $TMPD^+$ ("Würster's Blue").

of ascorbate, TMPD and dithionite. Fig. 2B shows the visible spectra of the species produced in reduced *minus* oxidized difference format. After poising the system with ferri/ferrocyanide only the strong absorbance band of a_3CN can be seen at 590 nm. In the ascorbate-ferrocyanide induced steady state, full reduction of the a_3 heme and partial reduction of the *b* heme are achieved. If anaerobiosis is achieved with TMPD as additional substrate, or by addition of dithionite, the *b* heme is fully reduced and the a_3CN a-peak is shifted by about 1 nm to the red.

An analogous shift of the Soret peak of the ferric mammalian cytochrome *c* oxidase cyanide complex occurs when the other centres in that enzyme are reduced to give a three-electron reduced state (not shown). We presume that the key event in each case is the reduction of the coupled CuB metal centre. However, in the case of the mammalian enzyme the binuclear a_3FeHCNCuB centre is changing from the ferric-cupric to the ferric-cuprous state. In the case of the *Thermus* enzyme the change is from the ferrous-cupric to the ferrous-cuprous state. As previously reported by Oertling et al. [18] this involves a change from a species with an epr signal characteristic of cupric CuB to a silent form, whereas with the mammalian enzyme it involves a change from an epr-silent species to one with a signal characteristic of low spin ferric iron (not shown). Although a similar classical oxidase cyanide complex can be obtained by direct cyanide addition to fully reduced aa_3 the intensity of the ba_3 absorbance is much higher than that of the mammalian enzyme and must indicate a different electronic structure even in the ferrous-cuprous state.

That the key change involved in cytochrome ba_3 is a one-electron process, something that the senior author was very doubtful about when he saw the initial reports, is also confirmed by a redox titration. Fig. 2C summarises the results of an experiment analogous to that shown in Fig. 2A, in which the redox level of the a_3CN centre is plotted as a function of the redox potential of the system determined from the ferro/ferricyanide ratio. An approximately straight line in the logarithmic plot with a slope close to 60 mV confirms the involvement of a single electron in the change from the ground (ferric ba_3) to the inhibited (ba_3CN) state.

If cyanide traps the binuclear centre in a mixed valence state with the opposite electron distribution to that trapped in classical cytochrome oxidase, what happens in the uninhibited aerobic steady state? Fig. 3 presents the preliminary results of a steady state study with ascorbate-TMPD as substrate. Similar results are seen with presumably physiological ascorbate-reduced cytochrome *c*-552 [22,23] as the substrate system (not shown). Fig. 3A shows the time course of oxidase reduction monitored at four wavelength pairs, two characteristic of heme *b* (one Soret and one visible region) and two characteristic of heme a_3 (one in the Soret and one in the visible region). The aerobic heme *b* steady state under the conditions shown is about 25% reduced and identical at all wavelengths. The a_3 heme shows a partial aerobic reduction more substantial in the visible than the Soret region, but significant in both, under conditions in which the mammalian enzyme would show almost complete oxidation of the a_3 heme until near anaerobiosis had been reached.

The difference spectra in steady state and after anaerobiosis are shown in Fig. 3B. The percentage of reduction of the a_3 heme is as great as or slightly greater than that of the *b* heme as measured in the Soret region. The same is true in the visible region where, however, the shape of the absorbance band shows characteristic differences from the corresponding absorbance of the fully reduced (anaerobic) species. Once again we are seeing, here under

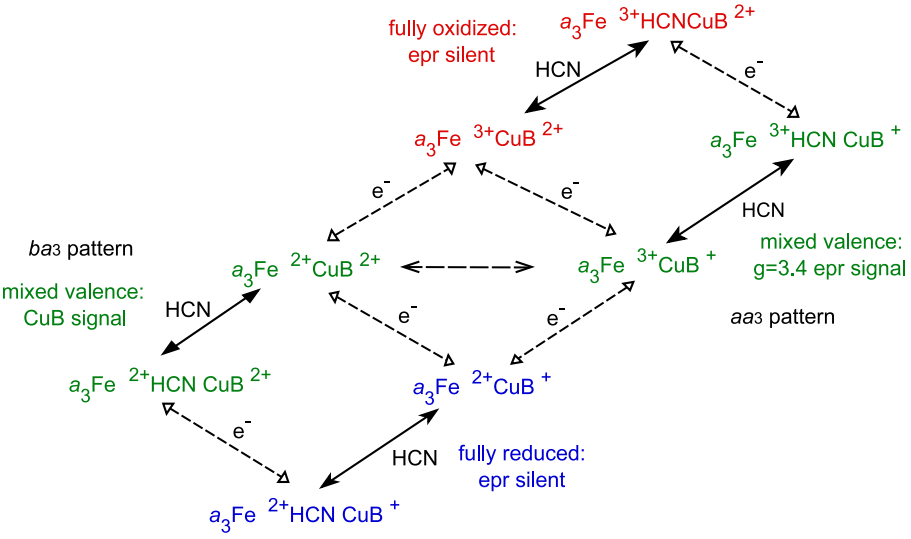

Fig. 4. Possible redox states of an oxidase binuclear centre during reduction in presence and absence of cyanide as an inhibitory ligand: comparison of the proposed aa_3 and ba_3 patterns. The central diamond shows the four possible functional redox states involved and the upper and lower cyanide-dependent equilibria, the species contributing to cyanide inhibition of the two enzyme types.

uninhibited conditions, a mixed valence state changing spectroscopically as anaerobiosis and reduction of the CuB component supervenes. It is also possible that the ba_3 steady state, unlike the classical aa_3 steady state, contains substantial concentrations of other intermediates in the reaction cycle (Fig. 1) including the 'P' and 'F' species.

How are these findings to be "unpacked" to fit into and extend the scheme of Fig. 1? Fig. 4 summarises the numerous possibilities that exist in going from a fully oxidized to a fully reduced binuclear centre (through the intermediates numbered 10, 11 and 1 within the "reductive" half cycle in Fig. 1). The ferric-cupric species can react with cyanide either slowly but tightly (aa_3) or poorly (ba_3). It can be reduced either to a ferric-cuprous state (aa_3) or to a ferrous-cupric state (ba_3), either of which species can react with cyanide to form a mixed valence cyanide complex. The mixed valence CN complex can be further reduced to a fully reduced cyanide complexed species either readily (ba_3) or with difficulty (aa_3).

It may be noted that the reported crystal structures for the aa_3 and ba_3 binuclear centres show very different proximal histidine εN-Fe distances [2–4,16] in the two enzymes (3.3 Å for ba_3 compared to $\approx$ 1.9Å for aa_3); but whether the unusually long Fe-N distance is preserved in ba_3 complexes such as that with cyanide is not known. As a thermophile *Thermus* cytochrome ba_3 functions in vivo at an elevated temperature, but despite the attempts of Giuffrè et al. [20] to explain some of their data in terms of a functionally modified temperature profile, its relationship with the structural, electron distribution and ligation differences also remains in need of further investigation.

4. Conclusion

In the aerobic steady state a substantial population of mixed valence binuclear centres (intermediate 11, Fig. 1) is probably present in both cytochromes aa_3 and ba_3. In ba_3 the electron is primarily on the iron atom, in aa_3 primarily on the copper B atom. It is therefore no longer possible to maintain as a generality the idea [6] that the last redox centre to be reduced in the oxidase is the a_3 Fe (cf. Fig. 4). Babcock was right to keep some of his options open. The world is more varied than we think, our knowledge is always provisional [24], and evolution can secure its adaptive "ends" in very different ways [25]. Those concerned with human affairs, as well as experimental scientists, must also keep this truth in mind.

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