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Review

'As prepared' forms of fully oxidised haem/Cu terminal oxidases

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1. Introduction

Members of the haem/Cu terminal oxidase superfamily catalyse the four electron reduction of dioxygen to water, and couple this exergonic reaction to the generation of a proton electrochemical gradient across the membranes in which they are embedded (see Refs. [1–8] for recent reviews). The superfamily can be divided according to function: some haem/Cu terminal oxidases accept electrons from cytochrome *c*, while the rest accept electrons from quinols. All haem/Cu terminal oxidases contain at least two haems and a redox-active copper centre (Cu_B); those that accept electrons from cytochrome *c* contain, in addition, a binuclear copper centre (Cu_A) which can accept

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Table 1
A glossary of terms frequently encountered in the cytochrome oxidase literature (after Palmer [7])

Fully oxidised:	$Cu_A^{2+} a^{3+} a_3^{3+} Cu_B^{2+}$ (655 nm and 830 nm absorption bands present)
Fast:	A preparation of enzyme that reacts (relatively) rapidly with cyanide. Also called 'rapid' and identical with 'pulsed' (Soret $\lambda_{max} = 424$ nm).
Slow:	A form of enzyme that reacts slowly with cyanide. Sometimes equated with 'resting' (Soret $\lambda_{max} = 417$ nm).
Resting:	Originally taken to be the enzyme as isolated but now also used to describe a preparation which contains a significant amount of the slow form.
Pulsed:	Enzyme subjected to a cycle of reduction and reoxidation under conditions which avoid the formation of turnover intermediates.
Partially oxidised:	$Cu_A^{2+} a^{3+} a_3^{3+} Cu_B^{2+}$ mostly (655 nm band partially present; 830 nm band present)
Oxygen-pulsed:	Enzyme subjected to cycle of reduction and re-oxygenation under conditions where turnover intermediates can form (Soret $\lambda_{max} = 428$ nm).
Oxygenated:	Identical with 'oxygen-pulsed'.
Reduced or fully reduced:	$Cu_A^{1+} a^{2+} a_3^{2+} Cu_B^{1+}$ (Soret $\lambda_{max} = 443$ nm; 655 nm and 830 nm bands absent)
Mixed-valence CO:	$Cu_A^{2+} a^{3+} a_3^{2+}$-CO Cu_B^{1+} (clearly split Soret; 655 nm band absent; 830 nm band present)
Mixed-valence formate:	$Cu_A^{1+} a^{2+} a_3^{3+}$-HCOOH-$Cu_B^{2+}$ (clearly split Soret; 655 nm band present; 830 nm band absent)
Mixed-valence cyanide:	$Cu_A^{1+} a^{2+} a_3^{3+}$-HCN Cu_B^{1+} (clearly split Soret; 655 nm and 830 nm bands absent)

The *closed* form refers to the fact that the *oxidised* enzyme reacts either slowly (*fast* form) or very slowly (*slow* form) with cyanide (Section 2.4). The *open* form refers to the fact that *partially reduced* enzyme reacts 10^4 times more rapidly with cyanide than the oxidised form [16–20].
N.B. The terms 'resting' and 'pulsed' have become ambiguous and their use should probably be dropped. Some authors use 'resting' to describe enzyme as prepared, which may contain only slow form, only fast form, or a mixture of the two, while others clearly equate 'resting' only with the 'slow' form. The term 'pulsed' is ambiguous because the precise method used for the 'pulsing' may or may not produce a mixture fully oxidised enzyme and intermediates in its turnover.

a single electron. All the eukaryotic haem/Cu terminal oxidases accept electrons from cytochrome *c* and contain haem A. Whilst there are many examples of prokaryotic haem/Cu oxidases that contain haem A, this is not the only haem type found, nor is its presence any indication that the enzyme in question is a cytochrome-*c* oxidase rather than a quinol oxidase. For instance, cytochrome *bo* from *Escherichia coli*, a quinol oxidase, contains haem types B and O [9,10] while cytochrome aa_3-600 from *Bacillus subtilis*, which is also a quinol oxidase, contains haem type A [11].

The most studied haem/Cu oxidase is bovine cytochrome-*c* oxidase (also called cytochrome aa_3). Of the thirteen polypeptides that comprise this enzyme, three, subunits I, II and III, are mitochondrially encoded [12]. Of these, subunit I contains the oxygen binding site, a binuclear centre composed of one of the haems, haem a_3, and Cu_B; the other haem, haem *a*, is also located in subunit I, while Cu_A, the primary acceptor of electrons from cytochrome *c* is located in subunit II. Subunit III contains no metal centres but together with subunits I and II, forms the catalytic core of the enzyme. It is this catalytic core that is essentially conserved throughout the haem/Cu oxidase superfamily, and while the homologues of some or all the remaining, nuclear-encoded, subunits may be present in other eukaryotes, these are not found in prokaryotes.

There are many types of heterogeneity known in haem/Cu oxidases. For example: isoforms of mammalian cytochrome-*c* oxidase that differ in one or more of the small nuclear-encoded subunits may occur within a single tissue [13], and recent studies on *E. coli* cytochrome *bo* have shown that the enzyme may assemble with Cu_B missing [14,15] or with either a type B or type O haem at the low-spin site [9,10]. In these examples the heterogeneity is essentially an immutable property of a given preparation of cytochrome *bo*. This review, however, is concerned with a type of heterogeneity where the forms, the so-called fast and slow forms, of the fully oxidised enzyme can be interconverted. For bovine cytochrome-*c* oxidase the phenomenon can be summarised as:

(a) There are at least two forms of fully oxidised bovine cytochrome-*c* oxidase in which the haem/Cu binuclear centre has different spectral, kinetic and ligand-binding properties.

(b) One of these, the fast form (see Table 1) [1], is equated with the fully oxidised form that is present in the normal turnover cycle of the enzyme.

[1] The current consensus view on the fast/slow phenomenon has come from the synthesis of accumulated evidence from different research groups using different experimental approaches. As a result of this, the nomenclature used by different groups to describe different manifestations of the same underlying process is different. This has caused, and still does cause, some confusion. The reader is, therefore, urged to consult Table 1 which attempts to explain some of this terminology.

(c) The other, the slow form, is considered to be an artefact that may appear during purification or on storage.

(d) The fast form converts spontaneously to the slow form, especially at low pH.

(e) The slow form may be converted back to the fast form by a cycle of reduction and reoxidation.

Heterogeneity in an enzyme preparation is certainly an experimental inconvenience, but it can also lead to misinterpretation of results. It is clearly important to be able to detect and, if possible, control heterogeneity, and, ultimately, to understand it at the molecular level. This review, therefore, sets out to answer, as far as possible, the following questions:

1. How can the fast and slow forms be identified?
2. How can the fast and slow forms be interconverted?
3. What is the molecular basis for the fast and slow forms?

2. Fast and slow oxidases: a comparison of spectroscopic and other properties

Some of the properties of fast and slow bovine cytochrome-*c* oxidase that have been used to distinguish the two forms are described in this section. Data for *E. coli* cytochrome *bo*, which also has fast and slow forms [21], are included for comparison. It is likely that the slow/fast phenomenon is a general property of at least the eukaryotic and bacterial members of the haem/Cu oxidase superfamily. Some examples where there is spectral or kinetic evidence for the presence of a slow form are: yeast (*Saccharomyces cerevisiae* [22]); shark (*Rhizoprionodon terranovae* and *Sphyrna lewini* [23]); cytochrome aa_3 from *Rhodobacter sphaeroides* [24,25]; cytochrome baa_3 from *Pseudomonas aeruginosa* [26]; and cytochromes *cao and* caa_3 from the thermophilic bacterium *Bacillus* PS3 [27,28]. Studies on Archaeal (Archaebacterial) haem/Cu terminal oxidases are in their infancy [1] and as yet there is no evidence for or against slow forms in this group of enzymes [29].

2.1. Electronic spectra

One of the first indications of heterogeneity in the binuclear centre of bovine cytochrome-*c* oxidase came from the wide range of Soret absorption maxima that were found for different preparations of the enzyme (418–424 nm; see, e.g., Ref. [30]). This variation arose from the presence of differing proportions of the slow and fast forms (and the Cl^--ligated form in some cases – see Section 3). The slow form has a distinctive asymmetric Soret absorption band with λ_{max} at about 417 nm caused by the superposition of two absorption bands: one at about 427 nm arising from low-spin haem *a*, and the other at about 414 nm arising from high-spin haem a_3 (Fig. 1A,B; see also Refs. [33–35]). The fast form of bovine oxidase has a broad but roughly symmetric Soret band with λ_{max} at about 424 nm (Fig. 1A). It seems likely that the Soret band of haem a_3 has two components in the fast form, one

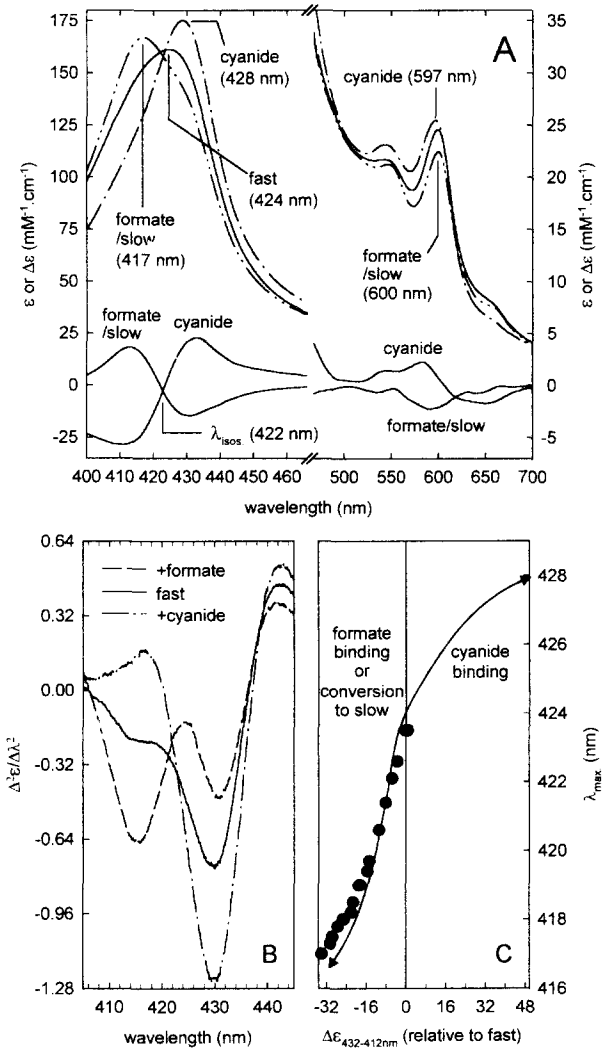

Fig. 1. Spectra of the fast, formate-ligated and cyanide-ligated forms of bovine cytochrome-*c* oxidase: the effects of cyanide binding, formate binding and conversion to the slow form on λ_{max}. (A, top section) Absolute spectra of bovine oxidase prepared by the method of Moody et al. [31] in 50 mM potassium phosphate buffer (pH 7.0), containing 0.5 mM potassium EDTA. The extinction coefficient at 422 nm is taken to be about 160 $mM^{-1} \cdot cm^{-1}$ [32]. The spectra, as indicated, are of the fast form (i.e., enzyme as prepared); the formate-ligated form (i.e., after 10 min incubation at 20°C with 20 mM potassium formate); and the cyanide-ligated form (i.e., after 6 min incubation at 20°C with 20 mM potassium cyanide). Note that the spectrum of the slow form is essentially identical to that of the formate-ligated form (Section 5.2). (A, bottom section) Difference spectra as indicated. (B) '2nd derivative' spectra derived as indicated from the absolute spectra in A (successive $\Delta\lambda$ values of 5 and 4.9 nm were used). (C, lines) The relationship between $\Delta\epsilon_{432-412\ nm}$, a direct measure of formate or cyanide binding to fast bovine oxidase, and λ_{max}, deduced from the absolute spectrum of the fast form and the difference spectra in (A). (C, symbols) Experimental measurements of $\Delta\epsilon_{432-412\ nm}$ versus λ_{max} taken during the spontaneous conversion ($k_{obs} = 1-1.5 \times 10^{-4}\ s^{-1}$) at 20°C of a stock solution (200 μM in 50 mM potassium bicine (pH 8.0), containing 1 mM potassium EDTA, 0.1% Tween 80 and 40 U/ml catalase) of fast bovine oxidase to the slow form.

at about 414 nm and the other at about 428 nm (Fig. 1B). This spectral behaviour probably results from there being an equilibrium between two spin-states of haem a_3 in fast bovine oxidase, but the magnetic properties and resonance Raman spectra of fast oxidase seem inconsistent with the presence of low-spin haem a_3 (Refs. [36] and [37]; see Section 6.3). The charge transfer band at about 661 nm in fast bovine oxidase clearly indicates the presence of high-spin haem a_3; this band is also seen in the slow form but at about 665 nm (from the '4th derivative' spectrum at pH 7.0, Moody, A.J., unpublished observation; see also Ref. [38] and the magnetic circular dichroism, MCD, data of Baker et al. [39]). When cyanide binds to haem a_3, λ_{max} shifts to 428 nm and the absorption band narrows. In this state both haems are low-spin and have almost coincident absorption in the Soret region (Fig. 1B).

The Soret absorption band of the slow form of *E. coli* cytochrome *bo*, where the low-spin haem, haem *b*, is either type B or type O and the high-spin haem, haem *o*, is type O [9], is similar in shape to that of the slow form of bovine oxidase, where both haems are type A, but shifted by about 11 nm to the blue (i.e., λ_{max} is about 406 nm [21]). Like the fast form of bovine oxidase, the fast form of *E. coli* cytochrome *bo* has a roughly symmetric Soret band, shifted to the red relative to the slow form, but here the difference between λ_{max} for the fast and slow forms is only 0.5–1 nm (λ_{max} for the fast form is 406.5–407 nm [40,41,21]). It seems that haem *o* is entirely high-spin in both forms of *E. coli* cytochrome *bo*. The charge transfer band associated with high-spin ferric haem *o* shifts from about 628 nm in fast cytochrome *bo* to about 638 nm in the slow form [21]. From the position of this charge transfer band in fast cytochrome *bo*, Cheesman et al. [41] have suggested that water is present as the distal ligand of haem *o* (the proximal ligand being His419 [42]).

The conversion of fast bovine oxidase to slow is characterised by the appearance of a difference spectrum in the Soret region with a peak at 413 nm and a trough at 431 nm, which accompanies the progressive shift in the position of λ_{max} to the blue. However, because of the contribution by haem *a* to the observed absorption band, and contrary to claims in the literature [35], the position of λ_{max} is *not* linearly related to the extent of the conversion (as monitored using $\Delta A_{432-412\ nm}$, for example; Fig. 1C). It should also be noted that the position of λ_{max} is affected by the accumulation of 'stable' turnover intermediates such as the E ('electronated'), P ('peroxy') and F ('ferryl') states. In the case of bovine cytochrome oxidase, samples of fast enzyme where λ_{max} is between 424 and 428 nm probably contain significant levels of the P and F states [31]. This is supported by the characteristic spectral changes [43] that occur in the visible region when such samples are incubated with catalase (Moody, A.J., unpublished observation). In the case of cytochrome *bo* the accumulation of turnover intermediates (e.g., 'F' ' [44]) can shift λ_{max} from 406.5 nm to as high as 410 nm.

2.2. Haem reduction kinetics

When dithionite is added to the fast form of bovine cytochrome oxidase, no clear kinetic distinction between the rates of reduction of the two haems is seen. In contrast, such a distinction is seen between the rates of reduction of the two haems in the slow form: haem *a* is reduced with a second order rate constant of about $8 \times 10^4\ M^{-1} \cdot s^{-1}$, whereas the rate of reduction of haem a_3 reaches a limit in the range 0.01–0.02 s^{-1} (see Ref. [45], and refs. therein). Contrary to earlier claims [46], it appears that this is a kinetic rather than a thermodynamic limitation on the reduction of haem a_3 since the rate of reduction is independent of the concentration of dithionite over a wide range (0.9–131 mM dithionite [2] [45], see also Ref. [48]), although it should be noted that reduction rates at least 10-fold greater than this limit can be obtained if either methyl viologen or benzyl viologen is used as reductant [49]. It may be that these reagents can bypass the limiting electron transfer step and donate electrons directly to the binuclear centre. The kinetic limitation seen with dithionite is not just on the reduction of haem a_3, but on the reduction of the binuclear centre as a whole; Cu_A and haem *a* are rapidly reducible in slow oxidase, while Cu_B and haem a_3 are not [45,50].

With the slow form of *E. coli* cytochrome *bo*, a similar limit on the rate of reduction of haem *o* by dithionite appears to be reached, provided that a low level of PMS is added to mediate electron transfer from dithionite to the enzyme [21].

The low kinetic limit on the intramolecular electron transfer to the binuclear centre in slow bovine oxidase is behind the observation of Antonini and co-workers [51] that the catalytic activity of 'resting' bovine oxidase, i.e., the enzyme as prepared, could be stimulated if it was reduced with dithionite and subsequently re-oxygenated. The immediate product of this procedure was called the 'oxygen-pulsed' form and is essentially synonymous with the 'oxygenated' form described by Okunuki and co-workers (see Ref. [52], and refs. therein), which has a Soret absorption maximum at 428 nm. However, rather than being a single species, the immediate product contains a mixture of 'stable' enzyme intermediates (e.g., the P an F states; [53]) which subsequently decay to give a fully oxidised product [54]. The activation seen by Antonini et al. resulted from the conversion of enzyme, which was largely in the slow form as prepared, to a mixture of the fast form and various intermediates in its turnover cycle.

2.3. Electron paramagnetic resonance (EPR) spectra

The slow form of bovine cytochrome-*c* oxidase shows characteristic EPR signals at $g' = 12$ and $g' = 2.96$ [55]

[2] Note that $SO_2^{\cdot -}$ is assumed to be the actual reductant [47], and that $[SO_2^{\cdot -}] = \sqrt{(K_{eq} \times [S_2O_4^{2-}])}$, where $K_{eq} = 0.85 \times 10^{-9}$ M.

that arise from the binuclear centre. Almost identical EPR signals are shown by the slow form of *E. coli* cytochrome *bo* at $g' = 13$, $g' = 3.2$ and $g' = 2.6$ [21]. The origin of these signals is thought to be an $S = 2$ spin system comprising ferric haem a_3 and cupric Cu_B. Some attempts to find a precise explanation for the $g' = 12$ signal [56–58] from bovine oxidase were hampered because the $g' = 2.96$ component was not taken into consideration. The simultaneous presence of the $g' = 12$ and $g' = 2.96$ signals had been reported previously [59,60], and, indeed, Greenaway et al. [59] suggested that they had a common origin because they had similar power saturation characteristics. Cooper and Salerno [55] have strengthened this suggestion and shown clearly that both signals arise from slow oxidase, and probably from the same spin system. Although the inclusion of the $g' = 2.96$ signal narrows the range of possibilities, the current level of spectroscopic knowledge is still insufficient for a definitive explanation to be made (see detailed discussions in Refs. [55] and [40]).

The binuclear centre of fast bovine oxidase is EPR silent apart from low and variable levels of a high-spin $g \approx 6$ signal that may arise from damaged enzyme [55], although transient signals at $g = 5$, 1.78 and 1.69 are seen when fully reduced enzyme is oxidised [61]. In contrast, the binuclear centre of the fast form of *E. coli* cytochrome *bo* is certainly not EPR silent, with signals at $g \approx 9$, $g = 3.74$ and $g = 3.08$ [40]. The EPR-silence of fast bovine oxidase and the EPR signals observed with fast cytochrome *bo* are all considered to be the result of magnetic interactions between the two metal centres. The precise nature of this spin coupling in each case is not certain, but it should be noted that the markedly different EPR behaviour of the fast forms of bovine oxidase and *E. coli* cytochrome *bo*, and, indeed, of the slow forms of both enzymes, does not necessarily reflect a great difference in binuclear centre structure [40].

2.4. Cyanide-binding kinetics

Cyanide is a potent inhibitor of the haem/Cu terminal oxidases, where it binds to the iron of the binuclear centre haem, inducing it to go low spin. With the fully oxidised enzymes, it is likely that the cyanide forms a bridge between the iron and the copper in the binuclear centre (with the carbon co-ordinating the iron [62–64]). The rate of onset of inhibition of haem/Cu oxidases during turnover (5×10^3 $M^{-1} \cdot s^{-1}$ for bovine oxidase [18]; $> 10^3$ $M^{-1} \cdot s^{-1}$ for *E. coli* cytochrome *bo* [65]) is comparable to the rate of cyanide binding to other haemoproteins, such as yeast cytochrome *c* peroxidase [66]. The fully oxidised forms bind cyanide much more slowly (< 2 $M^{-1} \cdot s^{-1}$ for bovine oxidase [67]; < 96 $M^{-1} \cdot s^{-1}$ for *E. coli* cytochrome *bo* [68,65,21]). The rapid rate of onset of inhibition, at least for bovine oxidase, may be in part caused by rapid binding to the E state (single-electron-reduced state) which is populated during turnover [19,20]. Fast oxidases bind cyanide at easily measurable rates: e.g., values between 1 and 2 $M^{-1} \cdot s^{-1}$ have been reported for fast bovine oxidase [67,39,19]. However, the slow form of bovine oxidase binds cyanide about 100-fold more slowly than the fast form [67,39,31,69]. Indeed, it is probable that binding to slow bovine oxidase is immeasurably slow, and that the observed rates actually reflect the rate of conversion of slow enzyme to fast [67] which is at least in part induced by electrons arising from the addition of cyanide (see Section 4). The reported responses of the rate of binding to changes in the concentration of KCN [67,69,70] are entirely consistent with this possibility. For example, working at relatively high pH (7.8), where spontaneous conversion from the slow to the fast form might be expected to occur [35], Panda and Robinson [70] found that the rate of binding to the slow form is only slightly dependent on [KCN] and that the *y*-intercept of plots of k_{obs} versus [KCN] was about 1×10^{-4} s^{-1}. This 'basal' rate is comparable to the rates reported for the interconversion of the fast and slow forms, which are in the range $1–3 \times 10^{-4}$ s^{-1} ([69,31,35]; see Section 4).

2.5. Other ligand binding reactions

A range of ligands other than cyanide are also known to bind extremely slowly to the slow form of bovine oxidase when compared to the fast form. These are: fluoride, azide and nitric oxide [71], and chloride (Moody, A.J., unpublished observation). In addition, slow bovine oxidase is unreactive towards carbon monoxide, which, under aerobic conditions, converts fast oxidase to the 'peroxy' form [72] and under anaerobic conditions converts fast oxidase to the 'mixed-valence' CO-ligated form [71]. It is also unreactive towards hydrogen peroxide, which converts fast oxidase to a mixture of the 'peroxy' and 'ferryl' forms [71,43]. The slow form of *E. coli* cytochrome *bo* shows a similar lack of reactivity towards hydrogen peroxide [21], whereas an oxyferryl intermediate is formed when hydrogen peroxide reacts with the fast form [68,44]. It is likely, as discussed for cyanide in Section 2.4, that in all these cases any slow binding or slow reaction that is seen with the slow form reflects the slow conversion to the fast form rather than direct binding to or reaction with the slow form itself.

3. Cl^--ligated bovine cytochrome-*c* oxidase

Besides the fast and slow forms it is also possible for the Cl^--ligated form of bovine oxidase to be present in enzyme as prepared. Chloride reacts with fast bovine oxidase at low pH (e.g., 6.5) to produce a form of the enzyme which has a similar spectrum in the Soret region to that of the fast form, but has ligand-binding properties that are different to those of both the slow and fast forms (Fig. 2; Refs. [31,73]). Although it remains to be established whether chloride has an analogous effect on any of

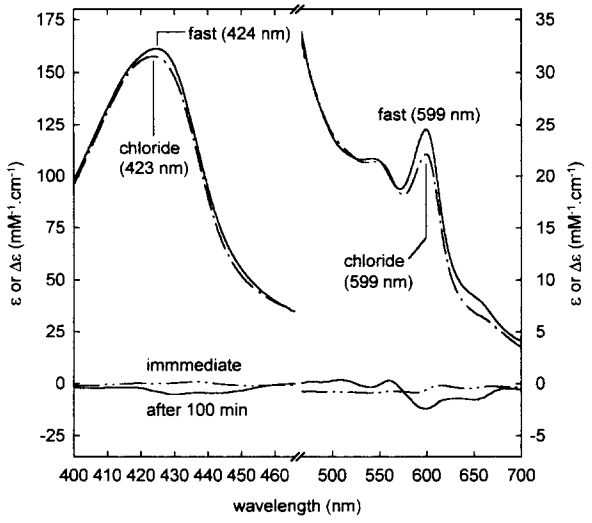

Fig. 2. Spectra of the fast and chloride-ligated forms of bovine cytochrome-*c* oxidase. (Top section) Absolute spectra of bovine oxidase prepared by the method of Moody et al. [31] in 50 mM potassium phosphate buffer (pH 7.0), containing 0.5 mM potassium EDTA. The extinction coefficient at 422 nm is taken to be about 160 $mM^{-1} \cdot cm^{-1}$ [32]. The spectra, as indicated, are of the fast form (i.e., enzyme as prepared) and the chloride-ligated form (i.e., after 100 min incubation at 20°C with 0.5 M potassium chloride). (Bottom section) Difference spectra as indicated, of enzyme immediately after dilution in buffer containing 0.5 M potassium chloride *minus* fast enzyme, and of enzyme after 100 min incubation in buffer containing 0.5 M potassium chloride *minus* fast enzyme.

the other members of the haem/Cu oxidase superfamily, nevertheless, it is clearly important to take account of this possibility in the design of experiments and purification protocols.

Some methods for the preparation of bovine oxidase have significant levels of chloride present at one or more stages, e.g., those of Fowler et al. [74]; Hartzell and Beinert [75] and Brandt et al. [76], and there is evidence from ligand-binding kinetics [73] and EPR spectra [77] that chloride is retained in enzyme prepared by the method of Brandt et al. [76]. One of the characteristics of chloride-ligated bovine oxidase is that it binds cyanide (at low pH, e.g., 6.5) at a rate intermediate between the rate of binding to the fast form and the apparent rate of binding to the slow form (Section 2.4, i.e., the apparent second order rate constant (at pH 6.5 and 22°C, with 32 mM KCN) is about 0.2 $M^{-1} \cdot s^{-1}$. The presence of phases of cyanide binding with 'intermediate' kinetics in various enzyme preparations has been noted by several authors [67,39,78], so it is certainly possible that the chloride-ligated form has been present in many cases. The following are two examples where this is likely to have been the case:

(a) The work of Chan and co-workers on the reaction of NO and fluoride with various preparations of cytochrome oxidase [79,80,56] showed that different preparations contain different proportions of subpopulations of enzyme that differ in their ligand-binding properties. Brudvig et al. [56] showed that the '$g' = 12$' EPR signal (Ref. [59]; see Section 2.3) arose from a subpopulation of enzyme that bound cyanide extremely slowly, which they termed the '$g12$' conformation, and which we now equate with the slow form of the enzyme. However, they also identified *two* other conformations of the oxidised enzyme, one that reacts with NO to give a high-spin haem a_3^{3+} EPR signal, which they called the 'resting' form, and the other that reacts with fluoride to give a high-spin haem a_3^{3+}-F^- EPR signal, which they called the 'oxygenated' form. The 'resting' conformation was only found in enzyme as prepared by the Hartzell/Beinert method, and after this enzyme was redox-cycled the product was the 'oxygenated' conformation, which, like the fast form [39,71], binds fluoride to give a characteristic set of EPR signals at $g = 8.5$, 6, 5,4.3 and 3.2. A similar set of EPR signals is seen when fluoride binds to the fast form of *E. coli* cytochrome *bo* [40]. Hence, it seems reasonable to equate the 'oxygenated' conformation of Brudvig et al. [56] with the fast form, and the 'resting' conformation with the Cl^--ligated form.

(b) In a recent study, Panda and Robinson [70] have reported the pH dependency for cyanide binding to enzyme prepared by a method based on that of Fowler et al. [74]. This dependency is similar to that of chloride-ligated oxidase in that the rate of cyanide binding increases by 3- to 4-fold as the pH is increased from 6.5 to 8.0 [31,73]. In contrast, the rate of binding to the fast form in the absence of chloride decreases slightly ($< 30\%$) over the same range [21].

4. Interconversion of the fast and slow forms

4.1. 'Spontaneous' interconversion

Baker et al. [39] found that bovine cytochrome-*c* oxidase, which in situ is presumably in the fast form, accumulates the slow form during purification procedures that involve low pH, and showed that the enzyme can be isolated in the fast form by using a procedure that avoids both low pH and prolonged dilution of the enzyme. When dilute at low pH they found that the fast form converted to the slow form, but at high pH, as a concentrated stock, the enzyme was essentially stable in the fast form. The need to avoid low pH during purification or storage to maintain the enzyme in the fast form is a general observation [76,31,81]. Papadopoulos et al. [35] examined the interconversion of the fast and slow forms by switching samples of enzyme between high and low pH (8.7 and 6.7, respectively) and monitoring the spectral changes that occurred. Starting with slow enzyme ($\lambda_{max} = 417.2$ nm) and going to high pH they observed a progressive red-shift in the maximum (to 422.9 nm over 2.5 h). Conversely, starting with relatively fast enzyme ($\lambda_{max} = 421.6$ nm) and going to low pH they observed a progressive blue-shift in the maximum (to

417.2 nm over 2.5 h). Although there is one aspect of this data that we have been unable to reproduce in our laboratory, i.e., the relatively rapid ($k = 2 \times 10^{-2}$ s^{-1}) and quite large spectral shift (about 50% of the total at 2–3 h) seen immediately following the pH change, we agree with the basic observation that there is some sort of pH-dependence in the interconversion of the fast and slow forms. The rate of this interconversion is found to be in the range $1–3 \times 10^{-4}$ s^{-1} [69,31,35]. Papadopoulos et al. [35] have interpreted this as a pH-dependent equilibrium between the fast and slow forms with a pK_a of about 7.8, but there are problems with this simple interpretation:

(1) The rate of approach to 'equilibrium' reported is essentially the same at both high (8.7) and low (6.7) pH. A pH-independent equilibration rate ($k_{eq} = k_{fast \rightarrow slow} + k_{slow \rightarrow fast}$) is inconsistent with a pH-dependent equilibrium ($K = k_{fast \rightarrow slow}/k_{slow \rightarrow fast}$) if the only involvement of pH is the requirement for the uptake of a proton during the conversion from fast to slow.

(2) The rate and extent of interconversion are highly dependent on both the preparation used and the precise conditions – buffer and detergent type, as well as pH – are important. For example, we have found that our fast enzyme, even when concentrated at relatively high pH (> 200 μM at pH 8.0), converts completely to the slow form in less than 24 h at 20°C (the data in Fig. 1C were obtained this way). This is in direct contrast to the observations of Baker et al. [39], and shows that there are factors involved other than pH or in addition to pH.

One of these factors may be 'electron leakage' from the medium. When bovine oxidase is diluted in some media a significant level of one-electron-reduced enzyme (E state) is found (which may be oxidised using ferricyanide [20,82]). In this case, enzyme that has converted to the slow form may be converted back to the fast form by transient reduction of the binuclear centre (Fig. 3), and a steady-state mixture of the fast and slow forms is obtained rather than an equilibrium mixture. Hence, to obtain a conversion from the fast to the slow form, it is sometimes necessary to include an electron 'sink', i.e., an oxidant

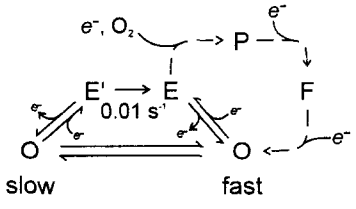

Fig. 3. A simple scheme to illustrate the potential effect of 'electron leakage' on the interconversion of the fast and slow forms of bovine oxidase. 'O' represents the fully oxidised forms (as indicated). 'E' is a single-electron-reduced form where the electron has equilibrated with haem *a* and Cu_A. Equilibration with the binuclear centre to form 'E' is limited by a slow intramolecular electron transfer step (Section 2.2). If 'E' is oxidised the fast enzyme is formed. However, if 'E' obtains a further electron and reacts with oxygen the stable oxygen intermediate 'P' is formed.

such as ferricyanide or hexachloroiridate [31]. A consequence of this is that the interconversion between the fast and slow forms may be affected by pH in a complex medium-dependent manner. 'Electron leakage' may also account for the shifts of λ_{max} to wavelengths > 424 nm seen by Papadopoulos et al. [35] which would arise from the slow accumulation of stable turnover intermediates such as the 'peroxy' (P) and 'ferryl' (F) states (Fig. 3).

The effect of detergent on the position of the Soret maximum of bovine oxidase has been noted [83,84], and this probably arises from differences in the relative proportions of the fast and slow forms. Although it is quite possible that this is a direct effect [35], it is worth noting that an indirect effect via detergent-dependent 'electron leakage' might also be responsible.

The conversion of fast *E. coli* cytochrome *bo* to the slow form seems to be more difficult to achieve that the equivalent conversion with bovine enzyme; simple incubation at low pH at 20°C is ineffective [40,65]. However, we have obtained conversion to the slow form by storing the enzyme at −20°C in buffer that has a pK_a with a positive temperature dependence, such as borate [21]. This method is also effective with the bovine enzyme [81], and can lead to inadvertent conversion of that enzyme to the slow form. A combination of factors may explain the success of this method with cytochrome *bo*: (1) low local pH in the frozen state, even though the pH may be high (e.g., 8.5) at ambient temperature; and (2) slowing or elimination of intermolecular electron transfer and/or 'electron leakage'.

4.2. *'Pulsing' methods*

Methods have been described for preparing either bovine cytochrome oxidase [39,31] or *E. coli* cytochrome *bo* [40] in the fast form. The presence of the Cl^--ligated form in addition to the fast form of the bovine enzyme (Section 3) can be avoided by using sulphate instead of chloride in the method of Brandt et al. [76].

Any preparation can be converted to the fast form by a redox cycle, but it is important to be sure that the enzyme is both fully reduced and fully reoxidised in this cycle. The term 'oxygen-pulsed' was originally coined by Antonini and colleagues [51] to describe bovine oxidase that had been reduced with dithionite and subsequently oxygenated, and which, as a result, showed an apparent increase in catalytic activity. However, as noted in Section 2.2, the product of this procedure is not a single species, rather, it is probably a mixture of the fast form of the enzyme and a number of 'stable' intermediates (i.e., the E, P and F states). In principle, enzyme prepared by reduction and subsequent oxidation in the absence of oxygen ('pulsed') should be identical with the fast form, since, in this case, the turnover intermediates cannot form, although, with hindsight, it seems likely that 'pulsed' enzyme prepared by Brunori et al. [85], by oxidation of reduced enzyme with anaerobic ferricyanide, must have contained significant

levels of intermediates since the Soret maximum was at 428 nm rather than 424 nm, the maximum found for the fast form (Section 2.1). Another approach, used by Kumar et al. [86,87], was to use a minimum of reductant to reduce the enzyme, so that all the enzyme completed a single turnover on oxygenation, but again, with hindsight, it seems likely that the '420 nm' form that Kumar et al. described still contained significant levels of the slow form, whose Soret maximum is at 417 nm (Section 2.1). A variation on this approach, that we have found to be successful (Moody, A.J., unpublished result), is to anaerobically reduce the enzyme using a slight excess of ascorbate and then to dilute it in aerobic buffer containing ascorbate oxidase. Since ascorbate is kinetically a poor reductant of cytochrome oxidase the excess of ascorbate can be removed in this way before further electron transfer can occur.

We have recently described a method for pulsing *E. coli* cytochrome *bo* [44,21] in which the enzyme is first reduced with ascorbate and 5-methylphenazinium methosulphate (PMS), and then dialysed under aerobic conditions. The presence of PMS enhances the rate of decay of any turnover intermediates that are left after oxygenation, although the precise mechanism by which this occurs is not known [21].

5. What is the nature of the fast / slow interconversion?

Current ideas on the nature of the interconversion between the fast and slow forms are concerned with the local structure of the binuclear centre and, in particular, the arrangement of the metal ligands. The notion that there is an endogenous ligand, the so-called 'slow' ligand, that is present in the slow form but displaced when the enzyme is converted to the fast form (either spontaneously or on transient reduction; Fig. 4) has arisen in part from extended X-ray absorption fine structure (EXAFS) studies (discussed next) and in part from the finding that formate binding induces changes in fast haem/Cu oxidases that

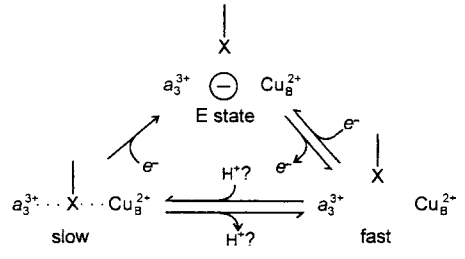

Fig. 4. A simple scheme to illustrate the idea of a 'slow' ligand. 'X' represents the unknown 'slow' ligand, which may ligate either or both metals in the binuclear centre. The interconversion of the fast and slow forms may involve a proton (see text for discussion). 'X' is displaced by an electron in the binuclear centre, to form the E state, and the product when the E state is oxidised is the fast form [i.e., O(slow) → E′ → E → O(fast) in Fig. 3].

almost exactly mimic their conversion to slow forms (discussed later in this section). The binding site for this ligand is variously seen as haem a_3 [31]; Cu_B [88] or a combination of haem a_3 and Cu_B (i.e., a bridge [48,89]). It should also be noted that other metalloenzymes, either haem-containing (e.g., cytochrome *c* peroxidase) or copper-containing (e.g., laccases) which interact with dioxygen or oxygen intermediates such as hydrogen peroxide, exhibit similar multiple-form phenomena to the haem/Cu oxidases, and that these are usually attributed to multiple ligation states of their metal centres (see Ref. [90] for a review).

5.1. Extended X-ray absorption fine structure studies (EXAFS)

EXAFS can potentially give direct information on the structure, i.e., ligand number, type and bond length, of the binuclear centre. There have been many EXAFS studies on bovine cytochrome oxidase (see, e.g. Refs. [89,67,91–93]; Refs. [94] and [95] are reviews), but although these studies have clearly confirmed that structural variability in the metal centres underlies the spectral and kinetic variability in oxidase preparations (Section 2; Refs. [67] and [91]), they have not been successful in revealing the precise structures that are involved. There are several reasons for this: (1) the inherent uncertainty in the method since EXAFS cannot distinguish between scattering atoms that differ by only a few atomic numbers; (2) the difficulty of deconvoluting contributions from multiple metal centres, e.g., Cu_B from Cu_A (the latter only recently was recognised to be a binuclear copper centre [96]); (3) the difficulty of deconvoluting contributions from multiple forms present in the same sample.

From studies on cytochrome oxidase prepared by the method of Yonetani [97,32], Powers et al. [89] presented evidence for a sulphur-containing ligand bridging between haem a_3 and Cu_B. Enzyme prepared by this method is largely in the slow form (see e.g., Ref. [67]). Hence, this sulphur-containing ligand is an obvious candidate for the slow ligand, a possibility that is further strengthened by its absence in enzyme that had been 'oxygen-pulsed' [98]. An attractive alternative possibility is that the bridging ligand could be chloride. Scott et al. [91] investigated the alternative possibility of a Cl^- bridging ligand (EXAFS effectively cannot distinguish between S and Cl) by comparing the Fe-EXAFS of two samples of enzyme, one prepared by the usual Hartzell/Beinert method [75], and the other by essentially the same method but in the absence of any chloride-containing buffers. They concluded that the bridge was absent in the chloride-free preparation, and hence, tentatively, that the bridge must be chloride. However, it is important to note the Hartzell/Beinert purification method normally involves chloride, and so, as noted in Section 3, it is possible that much of the enzyme used as a control in these experiments was in the Cl^--ligated form which could, in principle, be clearly distinguished from the slow

form on the basis of several spectral and kinetic criteria [31,73]. In contrast, the Yonetani purification method used by Powers et al. [89] does not involve chloride (but does involve at low pH step; see Section 4). Therefore, it is difficult to equate these two sets of observations.

This serves to highlight the principal problem with the interpretation of much of the EXAFS data that is currently available on haem/Cu terminal oxidases – the lack of additional spectral and kinetic data needed to establish the homogeneity and form of the preparation of enzyme being studied. Two recent studies on cytochrome aa_3-600 from *Bacillus subtilis* [99,100], which, as a quinol oxidase, lacks the binuclear Cu_A centre that is present in the bovine enzyme, further illustrate this. Powers et al. [99] found much the same behaviour as they found for the bovine enzyme [89], i.e., a bridging S or Cl ligand. In contrast, Fann et al. [100], using Cu EXAFS in conjunction with electron-nuclear double resonance (ENDOR), found no evidence for such a ligand. Among the samples examined by the latter group, was enzyme that had been purposely depleted of Cl^-, and which apparently contained none. This at least eliminates the possibility of a chloride-ligated form (see Section 3; although an effect of chloride on aa_3-600, like that found with the bovine enzyme, has not yet been demonstrated). Nevertheless, in both cases we have no information other than this on the form of the enzyme that was being used, and so have no means of properly assessing the significance of the difference in the results.

It is worth noting that the idea of a Cl^- bridging ligand in the slow form is not inconsistent with the observation that treatment of fast bovine oxidase with chloride does not yield the slow form [21]. As pointed out by Powers et al. [99], it is conceivable that Cl^- is already present as a Cu_B ligand in the fast form.

5.2. Formate-ligated oxidases: models for the slow oxidases

The addition of formate to the fast forms of bovine cytochrome oxidase or *E. coli* cytochrome *bo* induces changes that almost exactly mimic the fast to slow form conversion [31,88,21]. In particular, of a wide range of reagents tested with fast bovine oxidase, only formate induces the appearance of a '$g' = 12$' EPR signal like that shown by slow enzyme ([88]; see Section 2.3 and Refs. [101], [40] and [102]). Indeed, it is important to note that there is no concrete evidence for any difference in the properties, both spectral and kinetic, of the formate-ligated and slow forms of bovine oxidase or of *E. coli* cytochrome *bo*. Schoonover and Palmer [88] reported that the Soret maximum of the slow form of bovine oxidase increased from 416 to 423 nm as the pH was raised from 6.0. to 8.1, apparently without loss of the $g' = 12$ EPR signal, whereas the Soret maximum of the formate-ligated enzyme showed little change, but the pH-dependency of the Soret maximum of the slow form is *not* a general observation (see, e.g., Fig. 7 in Ref. [48], where the maximum is in the range 417–418 nm over the pH range 6.5–8.5; also Moody, A.J., unpublished observations). Moody et al. [31] reported that formate induced a slight blue shift in the Soret band when added to slow bovine oxidase, but at least part of this could have been caused by the reaction of formate with a small fraction of fast form.

The EPR behaviour of slow oxidases (Section 2.3), although not fully understood, may be particularly sensitive to the precise conformation of the binuclear centre [55,40]. Given that this is so, the sameness of the EPR signals shown by formate-ligated and the slow forms of haem/Cu oxidases (together with the other spectral and kinetic similarities) implies either that formate is triggering the same structural change that occurs during the fast to slow conversion, and its presence thereafter is unimportant or that the slow ligand is closely related to formate and can gain access to the binuclear centre without perturbing the structure. The latter seems more likely because formate appears to be a bona fide binuclear centre ligand. Schoonover and Palmer [88] found a close-to-stoichiometric association between ^{14}C-labelled formate and bovine oxidase during the elution of the enzyme from a Biogel P6 chromatography column. Furthermore, a comparison between the magnetic-circular-dichroism properties of formate-treated *E. coli* cytochrome *bo*, and the properties of the formate/carboxylate complexes of other, widely studied protohaem-containing proteins, e.g., *met*-myoglobin, suggests direct ligation of formate to haem *o* [41].

By analogy with formate, a carboxylate residue is an obvious choice for the slow ligand [102]. However, this no longer seems likely for two reasons: (a) it has been shown [21] that the conversion from fast to slow can still occur in cytochrome *bo* isolated from an *E. coli* strain in which the only highly conserved carboxylate residue in a transmembrane span close to the binuclear centre has been mutated ($E_{286} \rightarrow Q$); and (b) it is clear from the recent crystal structures of *Paracoccus denitrificans* and bovine cytochrome-*c* oxidases (Refs. [103] and [104], respectively) that widespread structural rearrangements would be required for either this residue or other possible residues in extramembrane loop regions (e.g., D407, D256 and D135 in *E. coli*) to be the slow ligand. An attractive alternative possibility is that the slow ligand is a free fatty acid [31,102], but this remains to be tested.

It is possible to perform a redox titration in the presence of formate, whereas a redox titration that includes the slow form would be impracticable because the rate of interconversion of the fast and slow forms is too slow (see Section 4). Hence, it is possible to determine the effects that formate, and, by extrapolation, conversion to the slow form, have on the midpoint potentials of the metal centres. This may, in turn, suggest something about the binding site of both formate and the slow ligand. Kojima and Palmer [105] used magnetic-circular-dichroism (MCD) to monitor

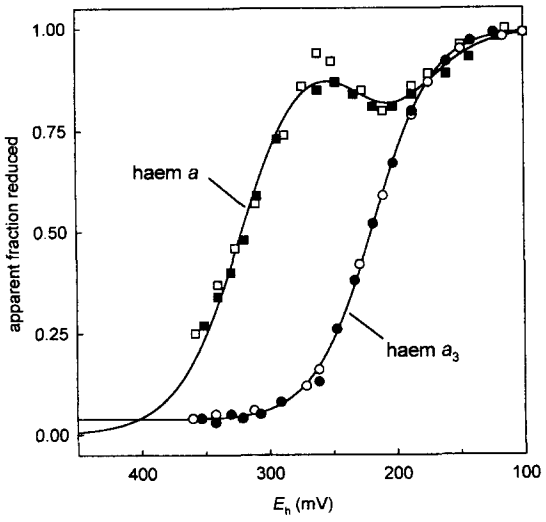

Fig. 5. Potentiometric behaviour of the haems of bovine cytochrome-*c* oxidase in the presence of 0.1 M formate. A replot of data from Kojima and Palmer [105] with 'best fit' simulations superimposed. The simulation procedure described in Moody and Rich [106] was used with a simplified model that includes interactions between haems *a* and a_3, and Cu_B, but not Cu_A. Of the seven microscopic midpoint potentials used four are mutually dependent during the fitting procedure (in other words the model is 'over-parameterized'). Three potentials are well-defined and are referred to in the text. These are (a) the E_m for haem *a* when the other centres are oxidised (high asymptotic potential); (b) the E_m for haem *a* when the other centres are reduced (low asymptotic potential); and (c) the E_m for haem a_3 when the other centres are reduced (low asymptotic potential).

the redox states of haems *a* and a_3 independently while titrating bovine oxidase in the presence of formate, and a replot of these data is shown in Fig. 5. To fully understand the effects of formate we need to fully understand the redox behaviour of the metal centres in the absence of formate. This behaviour is known to be complicated by anticooperative redox interactions between haem *a*, Cu_B and haem a_3 [107] and between Cu_A and haem *a* [108,106], and, as yet, a full quantitative description is unavailable. (This could require as many as fifteen microscopic midpoint potentials, i.e., $2^n - 1$, where n is the number of interacting components, which in this case is 4.) Nevertheless, a semi-quantitative assessment of the effects of formate can be made as follows.

Haem *a* is not *directly* affected by formate in that the high and low asymptotic midpoint potentials (320 and 200 mV, respectively) are similar to those seen in the absence of formate (Ref. [52] and refs. therein). The reversal in the level of reduction of haem *a* over the potential range 200 to 250 mV seen in the presence of formate is caused by an anticooperative interaction between haem *a* and an '$n > 1$' component with a midpoint potential in the same range, which is probably the binuclear centre acting co-operatively. The low asymptotic potential of haem a_3 is unaffected by formate, but the high asymptotic potential is considerably lowered, so that haem a_3 titrates essentially as an '$n = 1$' with a midpoint about 225 mV.

These effects of formate on the titration behaviour of bovine oxidase can be explained if formate binds only when the binuclear centre is fully oxidised. A possible explanation for this is that formate can form a bridge between haem a_3^{3+} and Cu_B^{2+}, which is displaced by reduction of either metal centre. However, carbon monoxide, which can ligate either haem a_3 or Cu_B *via* its carbon atom, and which does not form a bridge [109,110], binds only when the binuclear centre is fully reduced [111], and hence, like formate, affects the electron affinity of both haem a_3 and Cu_B, so bridge formation is not a necessity.

5.3. X-ray crystallography

The recent successes of two groups in solving the structures by X-ray crystallography to 2.8 Å of *Paracoccus denitrificans* [103] and bovine [104] cytochrome-*c* oxidase have added considerable clarity to the structural model that had been developed from structure prediction and studies on site-directed mutants [8,2,3]. Unfortunately, the study by Iwata et al. [103] does not shed any light on the specific structural difference between the fast and slow forms because azide, a binuclear centre ligand, was present during the crystallisation. Tsukihara et al. [104] found haem a_3 to be pentacoordinate, with Cu_B ligated by three histidine residues, and no evidence for a ligand bridging between haem a_3 and Cu_B, but there is uncertainty as to the form of the bovine enzyme that was present in their crystals. It is claimed to be the fast form because it showed monophasic cyanide-binding kinetics, but the rate of binding is not given and it is not stated whether the binding kinetics were measured before crystallisation or using redissolved crystals. This latter point is important because the crystals were prepared at pH 6.8 (Section 4.1). There is also the question of whether the fast form of the bovine enzyme is truly homogeneous (Section 6.2), so at the moment it is unclear how the results of Tsukihara et al. relate to the fast/slow question. In any case, a full picture will only emerge when the structure of the slow form has been solved.

6. Outstanding questions of detail

Notwithstanding the outstanding question of the structural basis for the fast/slow form phenomenon there are other outstanding questions concerning the detailed properties of the fast and slow forms.

6.1. Is there more than one slow form?

On closer inspection the dithionite-reduction kinetics of haem a_3 in slow bovine oxidase (Section 2.2) are found to be biphasic (e.g., 10–15% with $k = 0.025\ s^{-1}$, rest, $k = 0.005\ s^{-1}$ at pH 7.0 and 25°C [48]). This has also been found with the slow form of *E. coli* cytochrome *bo* [21].

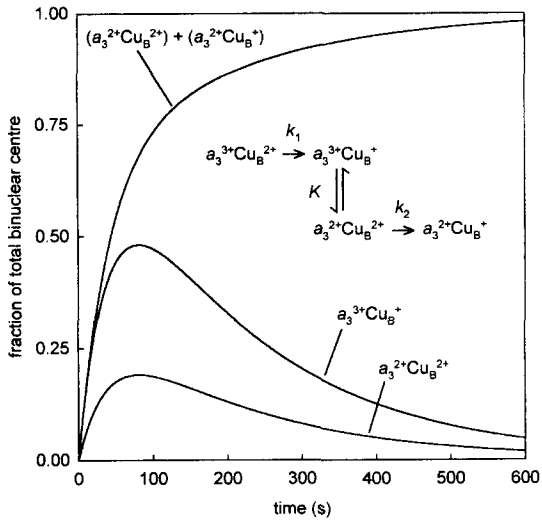

Fig. 6. A simulation of biphasic haem a_3 reduction by dithionite in slow bovine cytochrome-*c* oxidase using the model shown in the inset (see text for details). The primary parameters used are: $k_1 = 0.025\ s^{-1}$; $K = 0.4$; and $k_2 = 0.017\ s^{-1}$. However, the model can be reduced to the form A → B → C, where the rate constants for A → B and B → C are k_1 and $k_2 \times (K/1+K)$, i.e., $0.025\ s^{-1}$ and $0.0049\ s^{-1}$, respectively. It should be noted that the simulated time course of $(a_3^{2+}\ Cu_B^{2+}) + (a_3^{2+}\ Cu_B^{+})$ is equivalent to two *independent* exponential processes with the same values for the rate constants, where the contribution of the faster process is 11%. Hence, it corresponds well with the data of Cooper et al. [48] in the pH range 6.5–7.0.

Because of this biphasicity, Cooper et al. [48] suggested that there may be more than one slow form. They also argued that the disappearance of the $g' = 12$ EPR signal (Section 2.3) is only associated with the slower phase of haem a_3 reduction, and hence that only one of the two putative slow forms is responsible for this signal. Given the large error bars on their EPR data, that particular conclusion seems unwarranted, but it is certainly clear that the loss of the $g' = 12$ signal is not wholly associated with the faster phase of haem a_3 reduction.

It is worth noting that it is possible to explain the biphasic reduction kinetics using models in which there is only *one* slow form, if the two rate constants are seen as arising from the sequential reduction of the two components of the binuclear centre. The inset in Fig. 6 shows an example of such a model in which: (1) reduction of the binuclear centre can only occur via Cu_B; and (2) electron equilibration between Cu_B and haem a_3 is rapid, the equilibrium being shifted towards Cu_B at high pH. This particular example has the merit that it explains two other observations made by Cooper et al. [48]: (a) a decrease in the rate of the slow phase of haem a_3 reduction with increasing pH and (b) an increase in the extent of the fast phase of haem a_3 reduction with increasing pH.

With sequential reduction models the magnetic coupling between haem a_3 and Cu_B would be expected to be broken by the reduction of either component, thereby transiently revealing an EPR signal from the other component [112–114]. A transient signal from haem a_3^{3+} (high-spin $g = 6$) has been reported, but none from Cu_B^{2+} [45]. However, with the parameters used for the simulation in Fig. 6, less than 20% of the enzyme would be expected to show a signal from Cu_B^{2+}, and this could easily be missed.

6.2. *Is there more than one fast form?*

Although the fast form of bovine cytochrome oxidase is generally considered to be homogeneous on the basis of its reaction with cyanide [39], it has been suggested that this is not the case [31]. The observation that led to this suggestion is that formate binding (monitored, for example, using the formate-induced blue-shift in the Soret absorption band; Section 5.2) to the fast form is biphasic (Fig. 7A; Refs. [31,115,73,116]). This could be explained in terms of sequential processes involving a single population of enzyme [116]. However, taken as a whole the kinetic behaviour is much less consistent with this explanation, and suggests instead the presence of two subpopulations of fast enzyme that have different reactivity with formate, that have different spectra, and that are not in rapid equilibrium. This kinetic behaviour can be summarised as follows:

(a) In our hands, the rates of both phases in the formate-induced blue shift of the Soret band are dependent on the concentration of formate [3] (Fig. 7B). Baker and Gullo [114] concluded that the slow phase was concentration-independent, but this was on the basis of ambiguous results obtained at pH 8.8, where the rate of binding is low.

(b) The extents of $\Delta A_{432-412\ nm}$ resulting from the formate-induced blue shift in the Soret band for both phases are independent of the concentration of formate (same data as for Fig. 7B, details not shown).

(c) The extents of both phases are essentially pH-independent over the range 5.5–7.5 (Ref. [31] and Moody, A.J., unpublished observation).

(d) Full development of slow haem a_3 reduction kinetics (see Section 2.2) requires the completion of *both* phases in the blue-shift of the Soret band [31]. By monitoring the *complete* time course for appearance of slow haem a_3 reduction kinetics, and assuming that the relative extent of the slow phase of haem a_3 reduction is linearly related to the fraction of formate-ligated enzyme present, the subpopulation responsible for the slow phase of formate binding can be estimated to be about 26% of the total

[3] Nicholls [117] originally proposed, from measurements of the onset of inhibition at pHs 6.3 and 7.4, that the rate of formate binding to bovine oxidase was dependent on [formic acid]. Binding experiments (A.J. Moody, unpublished observations), which extend the pH range to 5.5, i.e. towards the pK_a of formic acid (3.77), for both bovine oxidase and *E. coli* cytochrome *bo*, are entirely consistent with this interpretation. In addition, Mitchell and Rich. [118] have shown that the net bound species is formic acid.

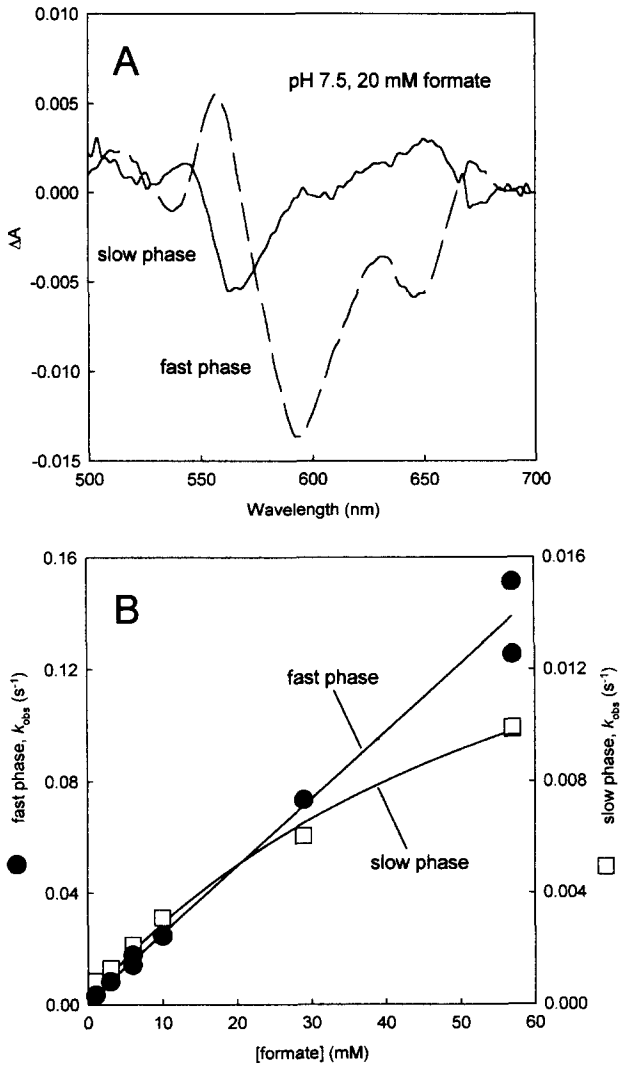

Fig. 7. Biphasic formate binding by fast bovine cytochrome-*c* oxidase. (A) A deconvolution of the spectral contributions in the visible region of the fast and slow phases of formate binding. Fast bovine oxidase was diluted to about 10 μM in 50 mM potassium phosphate (pH 7.5), containing 0.5 mM potassium EDTA and 1 mM potassium ferricyanide. The enzyme was incubated at 20°C for 10 min before a baseline scan was made. 20 mM potassium formate was then added and sequential scans taken every 14 s for about 9 min. The deconvolution is based on a fit of two independent exponentials to the time course of $\Delta A_{576-548\ nm}$ from which k_{obs} values of 0.026 s^{-1} and 0.0028 s^{-1} were obtained for the fast and slow phases, respectively. (B) The dependence of the rate of binding of formate to fast bovine oxidase on concentration of formate. Enzyme diluted to about 2.5 μM in 50 mM potassium phosphate (pH 7.0), containing 0.5 mM potassium EDTA was used. Binding was monitored using $\Delta A_{412-432\ nm}$. The second order rate constant for the fast phase is about 2.4 $M^{-1} \cdot s^{-1}$.

(Moody, A.J., unpublished observation; less reliable values of 26 and 31% were obtained by Moody et al. [31]).

(e) On reaction of fast enzyme with 0.5 M chloride at pH 6.5 (Section 3), both phases of formate binding progressively decrease in extent. However, the fast phase is lost completely in less than 90 min, whereas 4–5 h are required for the slow phase to completely disappear [73].

The presence of biphasic formate binding kinetics is not the only evidence that fast bovine oxidase is heterogeneous: Palmer and co-workers [39,71] have noted that nitric oxide does not seem to react uniformly with preparations of fast enzyme. Within 10 min incubation with about 1 atmosphere of NO a maximal EPR signal at $g = 6$ is obtained that amounts to about 0.2 equivalents of *high-spin* haem (per binuclear centre). Within the same time the Soret maximum shifts to 428 nm and there is a large decrease in the amplitude of the '655 nm' charge transfer band, consistent with the formation of a *low-spin* species (see Section 2.1 and Fig. 1). Palmer et al. [71] have suggested that about 20% of fast enzyme has a 'defect' at the Cu_B site that allows direct reaction with NO. This breaks the magnetic coupling in the binuclear centre thereby revealing an EPR signal from high-spin haem a_3^{3+}. In the remainder of the enzyme they suggest that NO forms a bridge between the two metal ions resulting in a low-spin haem species.

In contrast to the fast form of bovine cytochrome oxidase, there seems to be no question that the fast form of *E. coli* cytochrome *bo* is anything but homogeneous. All the ligands so far investigated, i.e., cyanide and hydrogen peroxide [68,44], and azide, fluoride and formate (Ref. [40] and Moody, A.J. and Mitchell, R., unpublished observations), appear to react monophasically.

6.3. What is the spin state of haem a_3 in fast bovine oxidase?

The slow forms of bovine cytochrome-*c* oxidase and of *E. coli* cytochrome *bo* are similar in most respects, despite the difference in haem type in the two enzymes, but there is a significant spectral difference between their fast forms (Section 2.1). While haem *o* in cytochrome *bo* is solely high-spin in the fast form, this does not seem to be the case for the bovine enzyme. One manifestation of this is that the formate and cyanide binding spectra for fast bovine oxidase are essentially the inverse of one another (except for the region around the '655 nm' charge transfer band; [119]). An explanation for this is that there is a mixture of low and high-spin haem a_3 in fast bovine oxidase, which goes completely high-spin when formate binds and completely low-spin when cyanide binds [119]. Although it seems certain the Soret band of haem a_3 has two components in the fast form (Section 2.1 and Fig. 1), there is, however, no evidence for low-spin haem a_3, e.g., from resonance Raman studies [37]. A recent comparison [36] of the magnetic susceptibility of the fast and slow forms of bovine oxidase may point to another explanation. Day et al. [36] found a clear difference between the two forms and proposed that the presence of some intermediate-spin haem a_3 in the fast form might account for this.

7. Overview

At present, by using a range of spectral and kinetic criteria, we can distinguish at least two types of fully oxidised bovine cytochrome-*c* oxidase, the so-called slow and fast forms. We can also show, by using the same sort of criteria, that cytochrome *bo* from *E. coli*, another member of the haem/Cu terminal oxidase superfamily, has slow and fast forms, even though there are some significant differences in detail between the bovine and *E. coli* enzymes. There are hints that the possibility of slow and fast forms is widespread, and perhaps ubiquitous, among haem/Cu oxidases of Eukaryotic and Bacterial origin, but as yet there is no evidence for such forms in a haem/Cu oxidase of Archaeal origin. Given the wealth of information that is being accumulated on a diverse range of haem/Cu oxidase types, it is to be expected that the question of just how general the slow/fast phenomenon is will be clarified soon. The same applies to the question of the precise structural basis for the difference between the two forms. This difference is likely to lie in the local structure of the binuclear haem/Cu site. In particular, the slow form is thought to be caused by the addition of or rearrangement of a ligand to one of the metal centres. Although much has been learned both indirectly (structure prediction and site-directed mutagenesis coupled with various kinetic and spectroscopic measurements) and directly (EXAFS and X-ray crystallography) about the binuclear centre structure in recent years, the identity of the slow ligand remains unknown. Whether the solution of these questions is only of passing interest to those concerned with the principal question, i.e., the general mechanism by which the haem/Cu oxidases couple proton translocation to electron transfer really depends on the prevalence of the slow/fast phenomenon. If, for instance, it turns out to be ubiquitous, then it is reasonable to suppose that it is an inevitable consequence of the structure required for these enzymes to fulfil their function, in which case, rather than simply being an experimental nuisance, it may also shed light on the general question of mechanism.

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