The reaction of halides with pulsed cytochrome *bo* from *Escherichia coli*

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Cytochrome *bo* forms complexes with chloride, bromide and iodide in which haem *o* remains high-spin and in which the '630 nm' charge-transfer band is red-shifted by 7–8 nm. The chloride and bromide complexes each have a characteristic set of integer-spin EPR signals arising from spin coupling between haem *o* and Cu_B. The rate and extent of chloride binding decreases as the pH increases from 5.5 to 8.5. At pH 5.5 the dissociation constant for chloride is 2 mM and the first-order

rate constant for dissociation is $2 \times 10^{-4} \text{ s}^{-1}$. The order of rate of binding, and of affinity, at pH 5.5 is chloride (1) > bromide (0.3) > iodide (0.1). It is suggested that the halides bind in the binuclear site but, unlike fluoride, they are not direct ligands of the iron of haem *o*. In addition, both the stability of the halide complexes and the rate of halide binding seem to be increased by the co-binding of a proton.

INTRODUCTION

Cytochrome *bo* from *Escherichia coli* is a member of the superfamily of terminal oxidases in which the site of oxygen binding and reduction is a haem/copper binuclear centre [1]. Both *E. coli* cytochrome *bo* and bovine cytochrome *c* oxidase, the most studied haem/copper oxidase, have at least two fully oxidized forms in which the binuclear centre has different ligand reactivities and spectroscopic properties (reviewed in [2]). For example, fluoride reacts with the fast form of both enzymes, resulting in complexes in which the haem iron is high-spin [3,4], whereas the slow forms are unreactive towards ligands [5,6]. Because of this low reactivity and the finding that slow forms appear only during purification or on storage of the purified enzyme, it seems that the slow forms are not physiologically relevant. There is evidence from the effects of chloride on the redox behaviour, the near-UV/visible spectrum, and the reactivity of the binuclear centre of bovine cytochrome *c* oxidase that chloride forms a complex with the fast form of this enzyme [2,7–9]. Subsequently we found that, 'as isolated', our preparations of *E. coli* cytochrome *bo* contained a subpopulation with properties analogous to those of the chloride-ligated bovine oxidase [10]. This was attributed to a fraction of fast enzyme that had bound chloride during purification. Because of this we adopted a purification procedure for *E. coli* cytochrome *bo* in which sulphate was intentionally substituted for chloride when preparing buffers [11]. More recently, Hirano et al. [12] have reported a 'novel' chloride-binding site in *E. coli* cytochrome *bo*. This observation was based on the different properties of preparations of cytochrome *bo* purified in the presence or absence of chloride ions. The purposes of this paper are (1) to report the effects of the halide series, chloride, bromide and iodide, on the electronic and EPR spectra of cytochrome *bo*, specifically on enzyme purified by a method that is well established as resulting in a homogeneous product that is both fully oxidized and fully in the fast form; and (2) to report the effect of pH on the kinetics of chloride binding to this enzyme. These and other results are

then discussed in the context of the results and interpretation presented by Hirano et al. [12]. A preliminary report of some of this work has appeared elsewhere [13].

EXPERIMENTAL

Growth of cells and membrane and enzyme purification

The *E. coli* strain RG145, which overexpresses cytochrome *bo* but lacks the other *E. coli* oxidase, cytochrome *bd*, was used [14]. Cell harvesting, disruption and preparation of membranes and purification of cytochrome *bo* were either as described by Moody and Rich [15] (enzyme used for kinetic measurements and electronic absorbance spectroscopy) or as described in Cheesman et al. [11] (enzyme used for EPR spectroscopy and electronic absorbance spectroscopy of the EPR samples). These methods are essentially identical, except that the final step used by Cheesman et al. [11] [to exchange the protein from 0.1 % (v/v) Triton X-100 into 0.2 % octyl β -D-glucopyranoside] was omitted by Moody and Rich [15]. To ensure that the enzyme was entirely in the fast form it was 'pulsed' (i.e. subjected to a cycle of complete reduction and complete re-oxidation) by the method of Moody and Rich [15]. Cytochrome *bo* prepared by this method has a Soret maximum between 406.5 and 407 nm; it reacts rapidly and essentially monophasically with H₂O₂, cyanide, azide, fluoride and formate [16,17]; and both haems are reduced rapidly by dithionite with phenazinium methosulphate as a mediator (more than 90 % of $\Delta A_{428-450}$ developed in less than 5 s with 10 mM sodium dithionite and 10 μ M phenazinium methosulphate at pH 7.0 and 20 °C [6]).

Halide binding measurements

Binding of halide to cytochrome *bo* was monitored by following halide-induced spectral changes with dual-wavelength spectro-

Abbreviations used: K_d , dissociation constant; k_{obs} , observed first-order rate constant for binding; k_{on} , second-order rate constant for binding; k_{off} , first-order rate constant for dissociation.

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scopy (on an in-house-constructed single-beam instrument; see below). Rapid cycles of measurements (cycle time less than 1 s) at 404, 420, 620 and 656 nm were made from which time-courses for the wavelength pairs 420–404 nm and 656–620 nm could be constructed. In all cases, absolute spectra of the enzyme were taken before halide addition and after completion of the time course of binding. The experimental design was as follows. A baseline scan between 390 and 700 nm was taken by using buffer alone (50 mM potassium Mes containing 0.5 mM potassium EDTA for pH < 7.0 or 50 mM potassium phosphate containing 0.5 mM potassium EDTA for pH ≥ 7.0). Purified enzyme (fast form; [6]) was diluted to 3–6 μM (ϵ_{406} was taken to be 233 mM⁻¹·cm⁻¹ [15]) in the buffer and a scan taken. The ligand was then added and the time course of binding was monitored, after which a final scan was taken.

Spectroscopy

Electronic absorbance measurements were made either on a single-beam spectrophotometer assembled in-house and constructed round a TM300 monochromator (Bentham Instruments) in which the gratings are mounted on a rotating turret driven by a microstepping motor (difference spectra and kinetic measurements), or on a Hitachi U-3000 double-beam spectrophotometer (absolute spectra). EPR spectra were recorded on an X-band ER-200D spectrometer (Bruker Spectrospin) interfaced to an ESP1600 computer and fitted with an ESR-9 liquid-helium-flow cryostat (Oxford Instruments).

RESULTS AND DISCUSSION

Effects of halides on the near-IR visible absorption spectrum of cytochrome *bo*

As judged by the changes induced in the electronic absorption spectrum of the enzyme, chloride, bromide and iodide all bind to fast cytochrome *bo*. Sulphate at the same ionic strength has no effect on the spectrum. Figure 1(A) shows absolute spectra in the Soret region of fast cytochrome *bo* before and after treatment with chloride. Chloride had a hypochromic effect on the Soret band effect (ϵ_{406} decreases by 10–11%), leading to a slight shift in the Soret maximum of 406.5 to 408.5 nm. Figure 1(B) shows the binding spectrum, i.e. halide complex minus ‘unligated’ enzyme, for chloride together with the binding spectrum of azide for comparison. In the wavelength range 500–700 nm the binding spectrum of chloride was almost identical with that of azide. The binding spectra of bromide and iodide (results not shown) were qualitatively the same as that of chloride. In the Soret region the binding spectra of chloride and azide were rather different; azide induced a red shift in the Soret band, in contrast with the hypochromic effect of chloride. The position of the ‘630 nm’ charge transfer band attributable to high-spin haem *o* was red-shifted by chloride, bromide and iodide. Its retention in all cases, which can be clearly seen from the absolute spectra shown in Figure 3(C), indicates that the halide complexes are all high spin. The pattern of spectral changes seen when chloride binds to cytochrome *bo* is qualitatively the same as those induced in fast bovine cytochrome *c* oxidase by chloride [2,8,9].

Kinetics of binding of chloride, bromide and iodide to cytochrome *bo*

When monitored in the Soret region, e.g. by using $\Delta A_{420-404}$, the kinetics of binding of chloride, bromide and iodide at low pH (6.0 or lower) are biphasic. With bromide and iodide there is an initial phase in which $\Delta A_{420-404}$ decreases (results not shown).

With chloride the first phase is more often seen as a lag before $\Delta A_{420-404}$ increases (Figure 2A) but a decrease with $\Delta \epsilon \approx 3 \text{ mM}^{-1} \cdot \text{cm}^{-1}$, i.e. comparable to that seen with bromide and iodide, can nevertheless be inferred from curve fitting by using two independent exponentials. Note that the increase in $\Delta A_{420-404}$ in the second phase is much more substantial ($\Delta \epsilon > 25 \text{ mM}^{-1} \cdot \text{cm}^{-1}$). In the range 500–700 nm, e.g. with $\Delta A_{656-620}$, the kinetics are monophasic (results not shown), and corresponds to the slower of the two kinetic phases seen in the Soret region. At higher pH (6.5 or higher) the kinetics are monophasic in both regions. The fast phase in the Soret region seen at low pH is probably caused by slight precipitation. Precipitation in our preparations of cytochrome *bo* is obvious below pH 5.5 anyway [10], and we have also observed chloride-induced precipitation of bovine cytochrome *c* oxidase at low pH (A. J. Moody, unpublished work). We have therefore ignored this phase and have assumed that only the slower phase reflects halide binding. Figure 2(B) shows the effect at pH 5.5 of chloride concentration on the observed rate constant, k_{obs} , for the slower phase (●) and on $\Delta \epsilon_{656-620}$ observed at equilibrium (○). If a rectangular hyperbola is fitted to the plot of $\Delta \epsilon_{656-620}$ against [KCl]:

$$\Delta \epsilon = \Delta \epsilon_{\infty} [\text{KCl}] / (K_d + [\text{KCl}]) \quad (1)$$

an apparent value ($2.1 \pm 0.3 \text{ mM}$) for the dissociation constant, K_d , of chloride is obtained. The corresponding plot of k_{obs} against [KCl] is non-linear. This behaviour could be interpreted as arising from a two-step reaction, where the first step is in rapid equilibrium [eqn. (2)], in which E represents the enzyme and L represents the ligand; in this case values for k_2 , k_{-2} and K_1 (i.e. k_{-1}/k_1) of $6.8 \times 10^{-3} \text{ s}^{-1}$, $4.3 \times 10^{-4} \text{ s}^{-1}$ and 109 mM respectively can be obtained by fitting eqn. (3). This gives an estimate for the overall dissociation constant, K_d [i.e. $K_1 \times (k_{-2}/k_2)$], of 6.9 mM which is in keeping with the value obtained above.

$$k_{\text{obs}} = k_2 [\text{KCl}] / (K_1 + [\text{KCl}]) + k_{-2} \quad (3)$$

However, because the concentrations used were well in excess of 1 mM we would expect non-linearity in the plot of k_{obs} against [KCl] to arise in any case from non-ideal solution behaviour of KCl [18]. Hence for simplicity we have assumed that this accounts for all of the non-linearity and that the binding of chloride is a one-step process. On this basis, with the constraint that $K_d = k_{\text{off}}/k_{\text{on}} = 2.1 \text{ mM}$, extrapolation to the y-axis gives a value for k_{off} of $2.1 \times 10^{-4} \text{ s}^{-1}$, and the initial slope gives a value for k_{on} of $0.10 \text{ M}^{-1} \cdot \text{s}^{-1}$.

The rates of binding of bromide and iodide are lower than the rate of binding of chloride. For example, at pH 5.5, with 115 mM halide, values of k_{obs} of 3.9×10^{-3} , 1.1×10^{-3} and $0.5 \times 10^{-3} \text{ s}^{-1}$ are obtained for KCl, NaBr and KI respectively. Under the same conditions, assuming that the spectral changes induced by chloride, bromide and iodide are quantitatively as well as qualitatively identical, the degree of saturation of binding is 98%, 88% and 78% respectively, giving estimates for k_{off} of 0.8×10^{-4} , 1.3×10^{-4} and $1.1 \times 10^{-4} \text{ s}^{-1}$ respectively. Hence it seems that k_{off} is essentially the same for all three halides. This suggests that a halide-independent factor controls k_{off} . A possibility is that this is the loss of a proton from the vicinity of the binding site as it seems that the affinity of the enzyme for chloride is increased by the co-binding of a proton (see below). Note that the apparent inconsistency between k_{off} for chloride obtained here and that obtained above by extrapolation of the plot of k_{obs} against [KCl] arises from the non-linearity of this plot. For

Figure 1 The effects of chloride on the electronic absorbance spectrum of fast *E. coli* cytochrome *bo*

(A) Absolute spectra in the Soret region of 'unligated' and chloride-ligated cytochrome *bo*. (B) Ligand binding spectra for chloride and, for comparison, azide, i.e. chloride-ligated minus 'unligated', and azide-ligated minus 'unligated', as indicated. For clarity the azide binding spectrum is displaced on the y -axis by $2.5 \text{ mM}^{-1} \cdot \text{cm}^{-1}$. To form the ligand complexes, fast enzyme (approx. $4 \mu\text{M}$) was incubated at 20°C either with 115 mM KCl for 25 min or with 2 mM NaN_3 for 10 s.

chloride, at least, the binding kinetics are not affected by the cation: LiCl, KCl and NH_4Cl all give the same result.

Effect of pH on the kinetics of chloride binding to cytochrome *bo*

Figure 2(C) shows the effect at 30 mM chloride of pH on k_{obs} and $\Delta\epsilon_{656-620}$. If we assume that the amplitude of $\Delta\epsilon_{656-620}$ at saturating chloride concentrations is independent of pH, then k_{obs} at pH 8.0 is consistent with a value of k_{off} of $5.0 \times 10^{-4} \text{ s}^{-1}$ (because $K = k_{\text{on}}/k_{\text{off}} = 0.49$ and $k_{\text{obs}} = k_{\text{on}} + k_{\text{off}}$). A value of k_{off} at pH 5.5 of $1.3 \times 10^{-4} \text{ s}^{-1}$ is obtained by using the same calculation. Hence it seems that the pattern of change in k_{obs} is caused by an approx. 4-fold increase in k_{off} as the pH increases from 5.5 to 8.0 superimposed on a decrease in k_{on} to approx. one-seventh. This behaviour is broadly consistent with both the affinity and rate of binding of chloride being increased by the co-binding of a proton, i.e. similar to the pattern we have discussed elsewhere for fluoride [17] and azide [17,19]. The pH dependence of binding of chloride to cytochrome *bo* is similar to that found for bovine cytochrome *c* oxidase [8].

Effect of chloride on cyanide binding kinetics

Chloride-ligated cytochrome *bo* binds cyanide at approx. 1/40 the rate of 'unligated' enzyme (fast form); with 2 mM KCN at

Figure 2 Kinetics of chloride binding to *E. coli* cytochrome *bo*

(A) Time courses of binding at pH 5.5 with 3.75, 7.5, 15, 30, 60 and 115 mM KCl (as indicated). The data have been corrected for the instantaneous increase in $A_{420-404}$ caused by the dilution of the enzyme on addition of the KCl solution at zero time. (B) Plots of final $\Delta\epsilon_{656-620}$ (○) and pseudo-first-order rate constants, k_{obs} , obtained from time courses of $\Delta\epsilon_{656-620}$ (●) against [KCl] at pH 5.5. (C) Plots of final $\Delta\epsilon_{656-620}$ (○) and pseudo-first-order rate constants (●) against pH with 30 mM KCl.

pH 7 k_{obs} is 0.0015 s^{-1} for chloride-ligated enzyme and 0.065 s^{-1} for 'unligated' enzyme. The rate constant for cyanide binding to the chloride-ligated enzyme exceeds the k_{obs} for chloride binding with 30 mM chloride at pH 7 (Figure 2C), so it must also exceed k_{off} for chloride at pH 7. Hence cyanide binding is not limited by the rate of chloride dissociation. It is possible that cyanide binding could enhance the rate of chloride dissociation if, as discussed above, the loss of a co-bound proton were involved, because HCN could directly replace HCl in the binuclear centre. There is no evidence to suggest that chloride and cyanide can bind simultaneously.

We have already suggested that some purified preparations of cytochrome *bo* might be partly in the chloride-ligated form [10]. A phase with 'intermediate' cyanide-binding kinetics ($k_{\text{obs}} = 0.024 \text{ s}^{-1}$ with 20 mM cyanide at pH 7.1) was observed that could be removed by redox-cycling the enzyme but was unaffected by treatment of the enzyme with formate. The results here are consistent with this suggestion; they are also reminiscent of the

Figure 3 EPR spectra (A, B) and electronic absorbance (C) of 'unligated' cytochrome *bo* and the bromide, chloride and fluoride complexes

Cytochrome *bo* at approx. 250 μM in 50 mM Hepes, pH 7.5, containing 0.2% octyl β -D-glucopyranoside was incubated overnight with 0.5 M KBr, 0.5 M KCl or 20 mM NaF. Complex formation in each case was confirmed by inspection of the electronic absorbance spectrum (C). The EPR conditions were: (A) temperature, 10 K; microwave power, 2.03 mW; modulation amplitude, 1 mT; and (B) temperature, 5 K; microwave power, 103 mW; modulation amplitude, 1 mT.

effects of chloride on cyanide binding to bovine cytochrome *c* oxidase (0.005 s^{-1} with 32 mM KCN at pH 6.5 with chloride bound, as opposed to approx. 0.05 s^{-1} without [8]).

Effects of fluoride, chloride and bromide on the EPR spectrum of cytochrome *bo*

Figure 3(A) shows the EPR spectrum of 'unligated' cytochrome *bo* together with spectra of the complexes with bromide, chloride and fluoride. The signals at $g = 2.98$, $g = 2.26$ and $g = 1.50$ due to low-spin ferric haem *b* are unchanged from the 'unligated' enzyme [11]. A feature at $g = 6$ probably arises in each case from a small percentage of protein (possibly damaged) in which the spin coupling between high-spin ferric haem *o* and Cu_B has been broken [6]. Chloride is exceptional in that it induces some rhombicity in this signal.

Of particular interest in the present context are the integer-spin signals that arise from enzyme in which the spin coupling between haem *o* and Cu_B is intact. These broad signals are seen most clearly under conditions of high microwave power and low temperature (5 K) (Figure 3B). Their essential features are retained not only in the presence of halides but also in the presence of formate, which binds to haem *o* but leaves it high-spin Fe(III) [20], and azide, which binds to Cu_B [19]. The observation of these signals depends critically on the spacings of the electronic ground-state energy levels, which are governed by parameters of the system such as the exchange coupling constant (J) and the zero-field splitting constant (D) at haem *o*. Therefore, in spite of the variable chemical nature of the ligands, the values of these parameters vary little between derivatives. Hence it is most unlikely that any one of these ligands is responsible for mediating the coupling between the unpaired electrons of $\text{Cu}(\text{II})$ and $\text{Fe}(\text{III})$ in the binuclear centre. There must exist an alternative route of spin-coupling between the two metals that might be intrinsic to the protein structure. We are currently simulating the binuclear centre EPR spectra, measured in both perpendicular

and parallel modes, by using a theoretical model that will also account for the novel temperature dependence that we have observed for magnetic CD features arising from haem *o*.

Relationship to the work of Hirano et al.

Orii et al. [21] examined the effects of chloride ions on haem-haem electron transfer in *E. coli* cytochrome *bo* and concluded that a bound chloride ion was required for normal haem-haem electron transfer and that depletion of chloride ions from the enzyme gave rise to complex kinetics in the reaction of fully reduced cytochrome *bo* with dioxygen. The complex oxygen kinetics observed by these authors has more recently been attributed to re-reduction of haem *b* by a tightly bound quinol [22,23]. In our view this interpretation is probably correct. Hence the likely effect of chloride is that it decreases the amount of bound quinone associated with a preparation, perhaps by competing for a binding site on the protein [22]. Subsequently the same group has proposed that a "novel" site on the enzyme modulates the binuclear centre in response to binding of chloride [12]. This conclusion was based on the observation that air-oxidized cytochrome *bo* prepared in buffers containing chloride exhibits an electronic absorption spectrum with a red-shifted Soret maximum compared with enzyme prepared in sulphate-containing buffers. At first sight this might seem consistent with the observations reported here. However, there are some clear differences in the results obtained by these authors and in their subsequent interpretation, both of which merit further discussion.

The assertion by Hirano et al. that the occupancy of the chloride-binding site controls the position of the Soret maximum is critically dependent on the three types of cytochrome *bo* preparation that they report as each containing a homogeneous binuclear centre. Two of these (designated UQO-409 and UQO-412) were prepared in the presence of chloride, whereas the third (UQO-407) was prepared by a protocol from which chloride had been excluded. On the basis of the position of the Soret maximum

(407 nm) and the X-band EPR spectrum (which contains a broad feature centred on $g = 3.7$) we conclude that this preparation probably contained a binuclear centre in the fast conformer. Unfortunately there is no additional information that might make this more certain, for instance monophasic kinetics for the reaction of the enzyme with H_2O_2 [6,15,16] and rapid reduction by dithionite with phenazine methosulphate as a mediator [6].

Incubation of either fast cytochrome *bo* (the present study) or UQO-407 with chloride leads to a small red-shift in the Soret maximum, which suggests the formation of the chloride-ligated form of the enzyme. Superficially, UQO-409 has similar optical properties, but careful inspection of the X-band EPR spectrum reveals a signal at $g = 3.14$ that is indicative of a subpopulation of the slow conformer [6], although it cannot be determined from the EPR spectrum shown whether UQO-409 also shows the broad feature around $g = 13$ that more clearly characterizes the slow forms of both *E. coli* cytochrome *bo* and bovine cytochrome *c* oxidase [6,8]. In contrast, there is no evidence for a subpopulation of slow enzyme in our chloride-ligated preparation, which shows a distinct signal at $g = 3.23$ but apparently nothing at $g = 3.14$ (Figures 3A and 3B).

Of the three preparations described by Hirano et al. [12], which by implication were presented as being homogeneous, UQO-412 seems most unlikely to be so. As discussed in detail elsewhere [2,6], our view is that preparations of cytochrome *bo* for which the Soret maximum is substantially red-shifted probably contain significant levels of 'stable' turnover intermediates such as oxyferryl species (e.g. F^+ [15,16]). Hirano et al. suggest that the difference between UQO-412 and UQO-409, which was prepared by "extensive washing" of UQO-412 by ultrafiltration, was the result of the loss of "diffusible factor(s)", but we interpret this instead as being caused by the slow decay of these intermediates. Hirano et al. state that "the variability of the Soret peak position cannot be attributed to the presence of the oxyferryl species in our preparations", but it is not clear what the evidence for this is. Although only the Soret region of the spectra of UQO-412 and UQO-409 is shown, this seems consistent with the interpretation that the blue shift in the Soret peak is caused by the decay of turnover intermediates because the (UQO-412) minus (UQO-409) difference spectrum (obtained by redrawing Figure 1 from [12]) has an appropriate maximum and minimum (around 420 and 402 nm respectively; see [15] for the difference in the spectrum of cytochrome *bo* before and after a burst of turnover with duroquinol).

Spin state of haem *o* in the halide complexes of cytochrome *bo*

Hirano et al. [12] interpret the changes in the electronic absorption spectrum of cytochrome UQO-407 on the addition of chloride as evidence for a transition of ferric haem *o* from high-spin to low-spin. In our view these changes are consistent with haem *o* remaining high-spin on chloride binding; in particular the '630 nm' charge-transfer band that is a marker for high-spin ferric haem *o* is still present after chloride binding (Figure 3C), although red-shifted as might be expected [24]. The derivative feature in the chloride-binding spectrum that results from this red shift (a minimum at 620 nm and a maximum at 656 nm; Figure 1B) is almost identical with that induced by azide (Figure 1B), which forms a high-spin complex in which haem *o* is high-spin [20], and is in marked contrast with the effect of cyanide, which forms a low-spin complex with haem *o* [4,10,25]. In addition, the magnetic CD spectrum of chloride-ligated cytochrome *bo* is consistent with the presence of only one low-spin ferric haem, and the negative band characteristic of high-spin

ferric haem *o* has moved to 647 nm [26], compared with 635 nm in the fast enzyme [27].

A physiological role for chloride binding?

Hirano et al. [12] have suggested a physiological role for chloride in the regulation of the activity of cytochrome *bo*. Their previous work purported to show that rapid intramolecular electron transfer in cytochrome *bo* requires the presence of chloride [21], which would support this notion, but all three of their enzyme preparations had similar activities (catalytic-centre activity of approx. 900 s^{-1}). Our fast enzyme, which is prepared and stored in the absence of chloride, shows catalytic-centre activities in excess of 1000 s^{-1} with decyl-ubiquinol as substrate [6]. In view of this, the involvement of the chloride-binding site on cytochrome *bo* described here and elsewhere [12,13] in normal turnover seems unlikely.

Where do chloride, bromide and iodide bind?

The results presented here confirm and extend the finding that chloride, bromide and iodide can bind to *E. coli* cytochrome *bo* [12,13], and the binding of chloride to bovine cytochrome *c* oxidase has already been demonstrated [2,8,9]. It is reasonable to suppose that chloride binding is a widespread phenomenon in haem/Cu oxidases, and that the chloride-ligated forms will have properties that are markedly different from those of the 'unligated' forms (e.g. different cyanide-binding kinetics). As we have already pointed out [2], a good strategy when purifying haem/copper oxidases is to avoid using media that contain chloride (see, for example, [11]). The red shift in the '630 nm' charge transfer band (Figures 1B and 3C) and the similarity in the binding spectra of chloride, bromide and iodide suggest that they have a common binding site within the binuclear centre, although they are not necessarily direct ligands of the haem *o* iron. Indeed, EXAFS studies on the chloride-ligated form of bovine cytochrome *c* oxidase (R. Sinclair, L. Powers, A. J. Moody and P. R. Rich, unpublished work) suggest that chloride is not a direct ligand of either the copper or the iron in the binuclear centre, although contributions in the copper-EXAFS due to the iron and in the iron-EXAFS due to the copper seen in the 'unligated' enzyme are lost in the chloride-ligated form. Nevertheless, Butler et al. [26] have found evidence that Cu_B in the binuclear centre of *E. coli* cytochrome *bo* can bind two molecules of NO and that chloride prevents the binding of the second molecule, which suggests that chloride might be a Cu_B ligand. This would seem to be in conflict with the EXAFS studies, but given the potential for ambiguity in the interpretation of EXAFS (see [2] for a discussion) of haem/copper oxidases it would be unwise to preclude chloride as a direct ligand to Cu_B on this basis.

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