‘CO₂-ligated’ cytochrome *c* oxidase: characterization and comparison with the Cl[−]-ligated enzyme

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A form of fully oxidized bovine heart cytochrome *c* oxidase that is induced by CO₂/HCO₃[−] is described. The ligand-binding properties of this form are similar to those of Cl[−]-ligated oxidase [Moody, Cooper and Rich (1991) *Biochim. Biophys. Acta* **1059**, 189–207]. Both bind cyanide at a rate (0.2 M^{−1}·s^{−1} at pH 6.5) intermediate between the rate of binding to the fast and slow forms of the enzyme, and binding of formate to both is almost undetectable. They are also similar in showing poor reactivity with H₂O₂, or with CO in the presence of O₂, which, with fast oxidase, induce the formation of the ‘ferryl’ and ‘peroxy’ states

respectively. However, there is a clear difference in the near-u.v./visible absorption spectra of the two forms; that induced by CO₂/HCO₃[−] has a Soret maximum at 427 nm whereas Cl[−]-ligated oxidase has a Soret maximum similar to that of fast oxidase at about 424 nm. It appears that both CO₂/HCO₃[−] and Cl[−] are members of a class of ligands that lowers the reactivity of the binuclear centre but does not impede intramolecular electron transfer from haem *a* to the binuclear centre, unlike the putative endogenous ligand responsible for slow oxidase.

INTRODUCTION

Cytochrome *c* oxidase (EC 1.9.3.1), the terminal component of the mammalian respiratory chain, catalyses the four-electron reduction of dioxygen. Electrons are transferred from cytochrome *c*, on the cytosolic side of the inner mitochondrial membrane, via Cu_A and haem *a*, to a binuclear metal centre, consisting of haem *a*₃ and Cu_B, where oxygen is bound and reduced. The reduction of oxygen is coupled to proton translocation from the matrix to the cytosol, with an average stoichiometry of 1 H⁺/e [1,2].

The problem of heterogeneity in fully oxidized preparations of bovine heart cytochrome *c* oxidase has long been recognized [3]. Fast oxidase, which has a Soret absorption maximum at 424 nm, is thought to be the form of the fully oxidized enzyme that is present *in situ* in the inner mitochondrial membrane, whereas slow oxidase, which has a Soret maximum at 417 nm, is found to accumulate during and after purification if the pH is low [4,5]. In contrast to fast oxidase, the binuclear centre of slow oxidase is difficult to reduce and shows poor reactivity with inhibitory ligands such as cyanide and CO [4,6]. Because the spectral and ligand-binding properties of enzyme ligated with another inhibitory ligand, formate, are almost identical to those of slow oxidase, the idea has arisen that the differences between fast and slow oxidase lie in a rearrangement of the binuclear centre in which an endogenous ligand, e.g. a carboxylate residue, occupies a similar spatial position to formate [5,7]. We have previously reported a further form of fully oxidized cytochrome oxidase that is induced when fast oxidase is incubated with Cl[−] [5]. In the present paper we describe another form that is induced when fast oxidase is incubated with CO₂/HCO₃[−], and compare this with that induced by Cl[−].

MATERIALS AND METHODS

Enzyme preparations

Two of the cytochrome oxidase preparations (Preparations 1 and

2) were made from bovine heart in the same way as Preparation C in Moody et al. [5]. This type of preparation, particularly when fresh, is essentially in the fast form, but Preparation 1, when used, contained 14% slow form. A third cytochrome oxidase preparation, Preparation 3, made by the method of Brandt et al. [8], was also used, and this contained no detectable slow form. All three preparations were stored in liquid nitrogen before use. Enzyme concentrations were estimated using *A*₄₂₂ ~ 160 mM^{−1}·cm^{−1} [9].

Generation of solid and gaseous CO₂

Solid CO₂ was made using a Frigimat Junior dry-ice maker from Aldrich Chemical Co. Ltd. (Gillingham, Dorset, U.K.). We generated CO₂ gas by sublimation of the solid in a Thunberg tube connected to Esco Butyl XX rubber tubing (Bibby Sterilin Ltd., Aldershot, Hampshire, U.K.). The gas was moistened by bubbling through water in line, and the supply was regulated by diverting part of the flow to an exhaust.

Incubation of cytochrome oxidase under a CO₂ atmosphere

Medium (200 mM Tris/200 mM Hepes, pH 8.0, containing 0.5 mM potassium EDTA) was placed at room temperature (18–22 °C) in a glass vessel fitted with a silicone rubber bung. The inlet for the CO₂ supply was formed by a hypodermic needle pushed through the bung and the outlet by a small vent in the bung. The system was flushed with gas for 10 min, during which time the medium was stirred. Stock enzyme solution (100–200 μM) was then diluted 10-fold in the medium, after which a slight positive pressure of CO₂ was maintained in the reaction vessel for 90 min. Samples could be removed during this time via the vent. The reaction vessel was then sealed for the remainder of the incubation. After a total of 18 h under the CO₂ atmosphere, the enzyme was concentrated 5–10-fold using a Centriprep-30 concentrator (Amicon Ltd., Stonehouse, Gloucestershire, U.K.) and stored in liquid nitrogen before use.

Although the pH of the medium starts at 8.0, this decreases as

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CO₂ dissolves and is hydrated (CO₂ + H₂O ⇌ H₂CO₃, followed by H₂CO₃ + OH⁻ ⇌ HCO₃⁻ + H₂O [10]). Over the first 10 min while the system is being flushed with gas, and before the enzyme is added, the pH decreases to 7.3. There is a further decrease to 6.8 over the first 90 min of incubation after the enzyme is added, i.e. while the slight positive pressure of CO₂ is being maintained, and thereafter when the vessel is sealed the pH is constant. Hence, during incubations of the enzyme with CO₂ the pH is in the range 6.8–7.3. The concentration of dissolved CO₂ when the medium is saturated is ~40 mM [11] and the concentration of HCO₃⁻ in equilibrium varies from ~80 mM at pH 6.8 to 250 mM at pH 7.3 taking the apparent pK_a for the equilibrium to be 6.5 [10].

Cyanide and formate binding kinetics

The kinetics of cyanide and formate binding at 20 °C were monitored using wavelength pairs in the Soret region: ΔA_{432–412} or ΔA_{428–410} were used interchangeably. These wavelengths were obtained by reference to binding spectra (e.g. cyanide [12] and formate [13]). Optical measurements were done either in a dual-wavelength spectrophotometer (Sigma Instruments, Berlin) or in an in-house-constructed single-beam spectrophotometer, under computer control, in which case for dual-wavelength measurements the monochromator was cycled between the wavelengths as described before [5].

Reagents

All reagents were supplied either by Sigma (Poole, Dorset, U.K.) or by Merck Ltd. (Lutterworth, Leicestershire, U.K.) and were

of Analar or similar grade, except for CO₂ (99.987 % pure) which was supplied by BOC Ltd. (Bristol, U.K.).

RESULTS

The effects of incubation with CO₂/HCO₃⁻ on cytochrome c oxidase

Spectral changes

Incubation with CO₂/HCO₃⁻ at pH 6.5–8.0, either by adding solid potassium bicarbonate or using a CO₂ atmosphere, leads to changes both in the ligand-binding properties and in the near-u.v./visible absorption spectrum of cytochrome oxidase. Figure 1 shows the effect of incubation of fast oxidase under a CO₂ atmosphere on the spectrum of the enzyme. The Soret absorption maximum shifts from 424 to 427 nm, and the resulting difference spectrum has a trough at 414 nm and a peak at 434 nm. The red-shift in the Soret maximum is accompanied by slight changes in absorbance in the visible region (for the maximum ΔA, ε < 1.5 mM⁻¹·cm⁻¹), while the '655 nm' charge-transfer band associated with high-spin ferric haem a₃ is unaffected by the treatment.

Cyanide binding

The spectral changes occur together with a decrease in the extent of the fast phase of cyanide binding by the enzyme (Figure 2) and the appearance of a new phase with slower kinetics. The pseudo-first-order rate constant, *k'* (20 mM KCN, pH 8.0), obtained by curve-fitting to the data in Figure 2 is 0.0040 ± 0.0001 s⁻¹ (mean ± S.E.M., *n* = 2). This is equivalent to a second-order rate constant of ~0.2 M⁻¹·s⁻¹, i.e. intermediate between the rate constants for cyanide binding to the fast and slow oxidase (1–2 and 0.01–0.02 M⁻¹·s⁻¹ respectively).

The rate of cyanide binding in the phase with intermediate kinetics induced by CO₂/HCO₃⁻ shows no significant pH dependence, values for *k'* of 0.0040 ± 0.0004 s⁻¹ (*n* = 3) and 0.0038 ± 0.0005 s⁻¹ (*n* = 3) being obtained at pH 6.5 and 8.0 respectively for the same batch of treated enzyme (using 20 mM KCN as in Figure 2). Similar changes are seen when HCO₃⁻ is used in place of CO₂, and these changes are retained even when the excess CO₂/HCO₃⁻ is removed by passage of the enzyme through a Sephadex G-25 column. They are also found when fast oxidase is stored frozen for 24 h in solid CO₂, unless a gas-tight container is used, although in this case there is also some spontaneous conversion to the slow form.

Formate binding

CO₂/HCO₃⁻ also induces distinct changes in the formate-binding properties of cytochrome oxidase (Figure 3). Fast oxidase shows two phases of formate binding, which differ by ~10-fold in rate (*k'* with 20 mM formate is ~0.1 s⁻¹ and ~0.01 s⁻¹ respectively at pH 6.5) [5]. Both of these phases decrease in extent on incubation with CO₂/HCO₃⁻, and they appear to be replaced by a third, much slower (*k'* < 0.0002 s⁻¹), phase.

Other reactions

Although difficult to assess quantitatively, it is clear that treatment of cytochrome oxidase with CO₂/HCO₃⁻ affects the reactivity of the enzyme towards both H₂O₂ and CO. High concentrations of H₂O₂ convert fast oxidase, via the 'peroxy' form, almost completely to the 'ferryl' form [15], whereas CO, in the presence of oxygen, converts fast oxidase almost completely

Figure 1 The effect of incubation under a CO₂ atmosphere on the near-u.v./visible absorption spectrum of cytochrome oxidase

Fast cytochrome oxidase was incubated at 20 °C under a CO₂ atmosphere for 18 h as described in the Materials and methods section. The absolute spectra (top of figure) are of control enzyme (---) and CO₂-treated enzyme (—) diluted to 2–3 μM in 50 mM potassium phosphate buffer, pH 6.5, containing 0.5 mM potassium EDTA, 40 μM potassium ferricyanide and 0.05 % n-octyl β-D-glucopyranoside. The difference between these spectra (bottom of figure) has been enlarged 8-fold.

Figure 2 The effect of incubation under a CO₂ atmosphere on the cyanide-binding kinetics of cytochrome oxidase

Fast cytochrome oxidase was incubated at 18 °C under a CO₂ atmosphere as described in the Materials and methods section. The starting pH was 7.3, and this decreased to 6.8 during the course of the incubation. (a) Time courses of cyanide binding to cytochrome oxidase after incubation of the enzyme with CO₂ for the times (*t*) indicated. Enzyme samples were diluted to 2–3 μM with 200 mM Tris/200 mM Hepes, pH 8.0, containing 0.5 mM potassium EDTA (the assay pH was 7.7–8.0), and the reaction was started by addition of 20 mM KCN. In each case the dead time before measurements were started was 5–10 s. (b) The extents of the fast and intermediate phases of cyanide binding versus incubation time. These extents were obtained by fitting two independent exponential phases and a linear phase to the time courses in (a) using the simplex method [14]. The linear phase was included because the enzyme preparation used here contained a significant level of the slow form (see the Introduction and Materials and methods sections). *K* for the slower exponential phase was constrained to 0.0040 s⁻¹, i.e. the average obtained from free fits to the time courses at 48.5 and 61.5 min.

to the 'peroxy' form [16]. Both of these forms have characteristic spectral features in the visible region; e.g. the difference spectrum of the 'ferryl' form versus the fast form has a peak at 580 nm, whereas the difference spectrum of the 'peroxy' form versus the fast form has a peak at 607 nm (Figure 4, B2 and A2 respectively). As we have seen, CO₂/HCO₃⁻ induces little change in the

Figure 3 A comparison of the cyanide- and formate-binding kinetics of cytochrome oxidase incubated under a CO₂ atmosphere with those of control enzyme

Fast cytochrome oxidase was incubated at 20 °C under a CO₂ atmosphere for 18 h as described in the Materials and methods section. Either this enzyme or untreated control enzyme (as indicated) was diluted to 2–3 μM in 50 mM potassium phosphate containing 0.5 mM potassium EDTA and 40 μM potassium ferricyanide. For the cyanide-binding kinetics the pH was 7.0 and the reaction was started by adding 20 mM KCN; for the formate-binding kinetics the pH was 6.5 and the reaction was started by adding 20 mM potassium formate. The dead time in each case was 5–10 s.

Figure 4 A comparison of the effects of CO and H₂O₂ on aerobic cytochrome oxidase that has been incubated under a CO₂ atmosphere with those of control enzyme

Fast cytochrome oxidase was incubated at 20 °C under a CO₂ atmosphere for 18 h as described in the Materials and Methods section. Either this enzyme (1) or untreated control enzyme (2) was diluted to 2–3 μM in 200 mM Tris/200 mM Hepes, pH 8.0, containing 0.5 mM potassium EDTA and 40 μM potassium ferricyanide. After 4 min the enzyme samples were treated either by directing a gentle stream of CO on the surface of the sample for 30 s while agitating it (A) or by adding 0.8 mM H₂O₂ (B). The Figure shows the differences between the spectra after treatment (5 min after for CO; 2 min after for H₂O₂) minus those before treatment.

Figure 5 The effect of incubation with Cl^- on the cyanide- and formate-binding kinetics of cytochrome oxidase

Fast cytochrome oxidase (Preparation B) was diluted to $1.7 \mu\text{M}$ in 50 mM potassium phosphate, pH 6.5, containing 0.5 mM potassium EDTA, $50 \mu\text{M}$ potassium ferricyanide, 120 i.u./ml catalase and 0.5 M KCl, and incubated at 22°C . (a) Time courses of cyanide and formate binding to cytochrome oxidase after incubation of the enzyme with Cl^- . Samples of the diluted enzyme were taken at the times indicated and the reaction started by addition of 20 mM potassium cyanide or formate. The dead time in each case is 5–10 s. (b) The extents of the fast ($\blacktriangle$) and intermediate ($\blacktriangledown$) phases of cyanide binding; the extents of the fast (∇) and slow ($\triangle$) phases of formate binding; and the total extent of formate binding ($\bullet$) versus incubation time. These extents were obtained by fitting two independent exponential phases to the time courses in (a) using the simplex method [15]. k' for the slower exponential phase of cyanide binding was constrained to 0.0025 s^{-1} , i.e. that obtained from a free fit to the time course at 30 min. For the formate binding data k' was constrained to 0.124 s^{-1} for the fast phase, and to 0.014 s^{-1} for the slow phase, i.e. those values obtained from a free fit to the time course at 2 min.

spectrum in the visible region. We would therefore expect to still be able to see the characteristic features of the 'peroxy' and 'ferryl' forms of the enzyme. However, the near absence of these features in the difference spectra shown in Figure 4 (A1 and B1,

Figure 6 A comparison of the cyanide- and formate-binding kinetics of fast cytochrome oxidase prepared by the method of Moody et al. [5] (Preparation 2) with those of cytochrome oxidase prepared by the method of Brandt et al. [8] (Preparation 3)

Stock enzyme was diluted to 2–3 μM in 50 mM potassium phosphate, pH 6.5, containing 0.5 mM potassium EDTA and $40 \mu\text{M}$ potassium ferricyanide. For the cyanide-binding kinetics the reaction was started by adding 20 mM KCN; for the formate-binding kinetics the reaction was started by adding 20 mM potassium formate. The dead time in each case was 5–10 s.

which correspond to the control spectra A2 and B2 respectively) shows that these reactions are almost absent in enzyme that has been incubated with $\text{CO}_2/\text{HCO}_3^-$.

A characteristic of fast cytochrome oxidase is that both haems are reduced rapidly and essentially simultaneously by 20 mM dithionite (at 20°C). With slow oxidase, under the same conditions, haem *a* is still reduced rapidly, but a period of 5–10 min is required for the complete reduction of haem *a*₃ [5]. The dithionite-reduction kinetics of $\text{CO}_2/\text{HCO}_3^-$ -treated oxidase appear to be no different to those of fast oxidase, at least with 20 mM dithionite.

Comparison with the effects of Cl^- on ligand binding by cytochrome *c* oxidase

We have already reported that incubation of fast oxidase with Cl^- leads to the appearance of a form of the enzyme that binds cyanide with intermediate kinetics, but which is spectrally similar to fast oxidase [5]. The rate of cyanide binding to Cl^- -ligated oxidase at pH 6.5 is similar to the rate of cyanide binding to the form of the enzyme induced by incubation with $\text{CO}_2/\text{HCO}_3^-$ that is described here. However, cyanide binding to Cl^- -ligated oxidase is pH-dependent, increasing almost 3-fold in rate between pH 6.5 and 8.0 (results not shown, but see Table II in [5]), whereas cyanide binding to the $\text{CO}_2/\text{HCO}_3^-$ -induced form is not. To allow further comparison of the properties of the two forms of the enzyme, we have examined the effect of Cl^- on the formate-binding kinetics of fast oxidase. Figure 5(a) shows the effect of preincubation with 0.5 M Cl^- at pH 6.5 on the cyanide- and formate-binding kinetics of fast cytochrome oxidase. As summarized in Figure 5(b), fast oxidase is converted almost entirely to the Cl^- -ligated form ($k' 0.0025 \text{ s}^{-1}$ with 20 mM KCN, pH 7.0) after incubation for 90 min. The changes in the formate-binding kinetics accompanying this conversion are marked; there is an

Table 1 The properties of CO₂/HCO₃⁻-treated cytochrome oxidase compared with those of the fast, slow and Cl⁻-ligated forms

Property	CO ₂ /HCO ₃ ⁻	Cl ⁻	Fast	Slow
Soret $\lambda_{\max}$ (nm)	427	422–424	424	417
Cyanide binding k' (M ⁻¹ ·s ⁻¹)	0.2	0.1–0.2 at pH 6.5	1–2	0.01–0.02
pH dependency	None	Positive	Negative	None
Formate binding k' (M ⁻¹ ·s ⁻¹)	< 0.01 at pH 6.5	Not detectable	Biphasic, 5 and 0.5 at pH 6.5	Unknown
Reaction with H ₂ O ₂	No	Unknown	Yes	No
Reaction with CO + O ₂	No	No	Yes	No
Haem reduction with 20 mM dithionite	Fast and simultaneous	Fast and simultaneous	Fast and simultaneous	Haem <i>a</i> fast; haem <i>a</i> ₃ , $k' \sim 0.01$ s ⁻¹

almost complete loss of the binding signal over the same time. Of the two phases of formate binding shown by our fast oxidase preparations [5], the fast phase is lost completely, whereas $\sim 20\%$ of the slow phase remains after 180 min incubation with 0.5 M Cl⁻. Prolonged incubation (> 4 –5 h) leads to the complete loss of detectable formate binding (results not shown). Like fast enzyme that has been incubated with CO₂/HCO₃⁻, enzyme that has been incubated with Cl⁻ shows no reactivity towards CO in the presence of oxygen (results not shown).

Cl⁻-ligated form present in cytochrome *c* oxidase as prepared

The method described by Brandt et al. [8] for the preparation of fast cytochrome oxidase involves the use of high concentrations of Cl⁻ at several stages. We have found that some preparations made using this method show a phase of cyanide binding with intermediate kinetics (Figure 6). The rate constant for this phase is clearly pH-dependent (results not shown), in the same way as the rate constant for the intermediate phase of cyanide binding induced by incubation of fast oxidase with Cl⁻ [5]. The extent of the formate-binding signal shown by this preparation is small compared with that shown by the other preparation shown in Figure 6, which was made by the method of Moody et al. [5], and that which is seen equates with the fast phase of cyanide binding. Thus, by implication, the subpopulation responsible for the intermediate phase of cyanide binding shows no detectable formate binding, again consistent with the properties of the Cl⁻-ligated enzyme.

DISCUSSION

In this paper we have shown that CO₂/HCO₃⁻ induces a novel form of bovine heart cytochrome *c* oxidase. This form binds cyanide at a rate intermediate between those of the fast and slow forms of the enzyme (0.2 M⁻¹·s⁻¹ compared with 1–2 M⁻¹·s⁻¹ and 0.01–0.02 M⁻¹·s⁻¹ for the fast and slow forms respectively). Since we have already observed the appearance of a form of the enzyme with similar cyanide-binding kinetics on incubation of cytochrome oxidase with Cl⁻ [5], the question immediately arises: is the form induced by CO₂/HCO₃⁻ the same as that induced by Cl⁻? The answer to this seems to be no, because, as we show here, the form induced by CO₂/HCO₃⁻ has several features that make it distinct from the form induced by Cl⁻ (Table 1).

One major difference is that the Soret absorption maximum of the CO₂/HCO₃⁻ form is close to 427 nm and hence is red-shifted relative to that of the fast form of the enzyme, which has a maximum at 424 nm, whereas the Soret band of the Cl⁻-induced

form is, if anything, slightly blue-shifted [5]. For the following reasons, we propose that the red shift in the Soret band induced by CO₂/HCO₃⁻ is due to haem *a*₃ rather than haem *a*. Haem *a*₃ in fast oxidase appears to have a split Soret band, with one component at ~ 414 nm and another at ~ 428 nm, whereas haem *a* has a single band at ~ 428 nm. We had suggested that a mixture of the high- and low-spin states of haem *a*₃ is present in the fast form of the enzyme, and that haem *a*₃ goes completely high-spin when formate binds (with $\lambda_{\max}$, 417 nm) or completely low-spin when cyanide binds (with $\lambda_{\max}$, 428 nm) [5]. However, a recent suggestion that there is a mixture of high- and intermediate-spin haem *a*₃ in fast oxidase [17] provides a better explanation, which is consistent with earlier magnetic c.d. and resonance Raman data [18,19]. Nevertheless, the notion of a split Soret band is still valid. If the red shift induced by CO₂/HCO₃⁻ is due to an increase in the intensity of the '428 nm' band of haem *a*₃, at the expense of the '414 nm' band, then we expect $\Delta\epsilon_{432-412}$ (or $\Delta\epsilon_{428-410}$) when cyanide binds to CO₂/HCO₃⁻-treated enzyme to be smaller than that found for the control enzyme. In fact, we expect $\Delta\epsilon_{432-412}$ to be approximately halved because $\Delta\epsilon_{432-412}$ for the CO₂/HCO₃⁻-induced change at 28 mM⁻¹·cm⁻¹ (Figure 1) is about half that (50 mM⁻¹·cm⁻¹) for cyanide binding to fast enzyme. This is indeed what is seen (Figures 2 and 3). However, if the red shift induced by CO₂/HCO₃⁻ were due to haem *a* we would expect no decrease in $\Delta\epsilon_{432-412}$, and, furthermore, the isosbestic point for the shift would be > 428 nm rather than 424 nm (Figure 1). In contrast, Cl⁻-treated enzyme, which does not have a red-shifted Soret band, binds cyanide with essentially the same $\Delta\epsilon_{432-412}$ as control enzyme [5].

Other differences between the CO₂/HCO₃⁻ and Cl⁻-induced forms of cytochrome oxidase relate to their ligand-binding kinetics. The rate of cyanide binding to Cl⁻-treated enzyme is markedly pH-dependent, increasing 3-fold between pH 6.5 and 8.0, but this is not the case for CO₂/HCO₃⁻-treated enzyme. It may also be that formate binding to CO₂/HCO₃⁻-treated enzyme, though slow, is detectable, since loss of the two phases of binding associated with fast enzyme seems to be associated with the appearance of a new phase of binding (Figure 3), whereas enzyme that has been treated at pH 6.5 with 0.5 M Cl⁻ for 4–5 h lacks any detectable formate binding (results not shown).

It is clear, therefore, that CO₂/HCO₃⁻ induces a novel form of cytochrome oxidase. However, the identity of the bound species (CO₂, H₂CO₃ or HCO₃⁻) and of its binding site are unknown. Given the general lowering of the reactivity of the binuclear centre (binding of both cyanide and formate is inhibited, as are the reactions with H₂O₂ and CO in the presence of O₂), it is likely that the binding site is close to the binuclear centre, although the

observation of a spectral shift in haem a_3 does not necessarily imply that the site directly involves the haem iron. The binding appears to be tight, since the effects of incubation with $\text{CO}_2/\text{HCO}_3^-$ are retained after removal of the excess by passing the enzyme down a Sephadex G-25 column, but it is still possible that there is no tightly bound species and that the presence of $\text{CO}_2/\text{HCO}_3^-$ triggers a change in the conformation of the binuclear centre that is retained in its absence. This latter possibility would seem more likely if Cl^- and $\text{CO}_2/\text{HCO}_3^-$ had identical effects on the enzyme, but the fact that their effects are only broadly similar suggests instead that they have a common tight binding site.

Phases of cyanide binding with intermediate kinetics have been seen by others in cytochrome oxidase as prepared [4,20], and so it is worth considering whether these could be caused by the presence of the $\text{CO}_2/\text{HCO}_3^-$ -ligated enzyme. However, at least in one case where we have observed a cyanide-binding phase with intermediate kinetics, i.e. in preparations made by the method of Brandt et al. [8] (Figure 6), it seems certain, in agreement with Lodder [21], that this is caused by the presence of the Cl^- -ligated form. Nevertheless, high levels of the $\text{CO}_2/\text{HCO}_3^-$ -induced form are certainly found after storage of the enzyme overnight in solid CO_2 , unless it is kept in a gas-tight container. With the recent interest in the various members of the superfamily of terminal oxidases that contain Cu/Fe binuclear centres [22] and to which mammalian cytochrome c oxidase belongs, it has become common practice to transport enzyme samples between laboratories, and often the only practical way to do this is by packing the samples in solid CO_2 . If other members of the superfamily are modified by storage in solid CO_2 in a similar way to mammalian cytochrome c oxidase, then this could lead to confusion about the characteristics of these enzymes, and in particular their ligand-binding properties. Hence, we advise the precautionary use of gas-tight containers for storage of such samples in solid CO_2 .

Both $\text{CO}_2/\text{HCO}_3^-$ and Cl^- clearly decrease the reactivity of the binuclear centre, but they do not impede intramolecular electron transfer from haem a to the binuclear centre, unlike the putative endogenous ligand present in the slow form of the enzyme [5]. The physiological concentrations of both $\text{CO}_2/\text{HCO}_3^-$ and Cl^- are probably millimolar or greater [23], and so both could be ligands *in vivo*. However, it appears that both $\text{CO}_2/\text{HCO}_3^-$ and Cl^- must be displaced during turnover because if this were not

the case it would not be so simple to prepare cytochrome oxidase that is wholly in the fast form [4,5]. It follows that both $\text{CO}_2/\text{HCO}_3^-$ and Cl^- are probably ineffective as inhibitors of cytochrome oxidase.

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