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Characterisation of 'fast' and 'slow' forms of bovine heart cytochrome-*c* oxidase

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We have prepared cytochrome-*c* oxidase from bovine heart (using a modification of the method of Kuboyama et al. (1972) J. Biol. Chem. 247, 6375–6383) which binds cyanide rapidly, shows no kinetic distinction between the two haems on reduction by dithionite, has a Soret absorption maximum above 424 nm, and has a negligible '$g' = 12$' EPR signal. On incubation at pH 6.5 this 'fast' oxidase reverts to the 'slow' ('resting') form characterised by slow cyanide binding, slow reduction of haem a_3 by dithionite, a blue-shifted Soret maximum and a large '$g' = 12$' signal. Incubation of 'fast' oxidase with formate produces a form of the enzyme with properties almost identical to those of 'slow' oxidase. The kinetics of formate binding to 'fast' oxidase are found to be biphasic, revealing the presence of at least two 'fast' subpopulations in our preparations. Evidence is presented that there is an equilibrium mixture of high-spin and low-spin forms of haem a_3 in both 'fast' subpopulations at room temperature. Incubation of 'fast' oxidase with chloride or bromide at pH 6.5 produces forms of oxidase with much lower rates of cyanide binding. Our working hypothesis is that formate mimics a binuclear centre ligand which is present in the 'slow' form of cytochrome oxidase. Although we show that chloride and bromide can also be ligands of the binuclear centre, possibly onto Cu_B, we can rule out either of these being the ligand present in the 'slow' enzyme. We will argue that the 'fast' and 'slow' forms of oxidase are equivalent to the 'pulsed' and 'resting' forms of oxidase, respectively.

Introduction

Cytochrome oxidase (EC 1.9.3.1) as isolated from bovine heart is generally heterogeneous, both within an individual preparation and between different preparations [1]. This heterogeneity can be revealed by examining the reactivity of a given enzyme preparation with inhibitory ligands [2,3]. Using cyanide as an example, it has been shown that most preparations exhibit two phases of cyanide binding to the fully-oxidised enzyme. These phases are spectrally indistinguishable and differ in rate by about two orders of magnitude [4]. In some preparations a third phase of cyanide binding with intermediate kinetics is seen [3,5,6], but generally most oxidase preparations can be considered to be mixtures of two types of enzyme which differ dramatically in their reactivity with a variety of haem a_3 ligands, i.e. 'fast' oxidase, which is relatively reactive, and 'slow' oxidase, which is particularly unreactive [1,6]. Furthermore, it is now known that the 'slow' form has at least two other characteristics that distinguish it from the 'fast' form, i.e., (1) an EPR signal, the '$g' = 12$' signal, which arises from the binuclear centre [2], and (2) a blue-shifted Soret absorption band [6]. It is worth noting here that the rate of cyanide binding even to 'fast' oxidase is slower by five orders of magnitude than the rate of onset of inhibition observed during turnover of the oxidase, and, to account for this, it has been suggested that cyanide may bind very rapidly to a partially-reduced intermediate in the reaction cycle [7,8]. This form of the enzyme is seen by some as having a different protein conformation and has been termed the 'open' form; the fully-oxidised 'fast' and 'slow' forms are by definition termed 'closed' forms [9].

Heterogeneity has also been revealed by the observation that many preparations of cytochrome oxidase are largely in an essentially inactive state, which is characterised by slow intramolecular electron transfer, Cu_A and haem *a* being rapidly reduced by added reductant, but haem a_3 and Cu_B being reduced much

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more slowly. This form of the enzyme, which is referred to as the 'resting' form, can be activated, e.g., by complete reduction followed by oxygenation [10], after which the active 'pulsed' form * of the enzyme slowly reverts to the 'resting' form. It has been suggested that the 'resting' and 'pulsed' forms of cytochrome oxidase are synonymous with the 'slow' and 'fast' ligand binding forms, respectively [11], and circumstantial evidence tends to support this idea. However, as yet there is little direct evidence, and, indeed, Palmer et al. [1] have argued that there is an essential difference between the 'pulsed' and 'fast' oxidase, perhaps at the level of a modified amino-acid, because the former reverts to the 'slow'/'resting' form, whereas the latter form does not.

There has been some speculation that the interconversion between the 'slow' and 'fast' forms of oxidase ** represents a control mechanism of physiological significance [12,13]. However, the discovery by Baker et al. [6] (and also Brandt et al. [11]) that oxidase could be isolated in an apparently homogeneous and stable 'fast' form, and the fact that, to our knowledge, the 'slow' form has not been observed in intact mitochondria, strongly suggests that the 'slow' form is simply an artefact of the preparation method. Nevertheless, the question as to the structural and mechanistic differences between the two forms of the enzyme remains of considerable interest, not only because the presence of the 'slow' form causes experimental difficulties, but also because of the relationship between ideas concerning the interconversion of the 'fast' and 'slow' forms, ideas concerning the interconversion of the 'open' and 'closed' forms, and ideas concerning the mechanism by which cytochrome oxidase translocates protons. It was suggested soon after the first description of the 'fast' form of the oxidase [10] that the difference between this form and the 'slow' form is, by analogy with haemoglobin, primarily 'polypeptide-based' (Ref. 12, see also Ref. 14). An essentially allosteric model, with the electron being the effector, was proposed. In this type of model the intramolecular electron transfer between haem a/Cu_A and the binuclear centre is seen as being kinetically limited, a low rate being observed in 'slow' oxidase because the transfer pathway is disrupted in this protein conformation. A similar model has been proposed to account for the difference between the 'closed' and 'open' forms, and it has been further suggested that these conformations might correspond to the electron input and output states of a redox-gated proton pump [15].

The observation that cytochrome *c* can stimulate the rate of the intramolecular electron transfer in 'slow' oxidase tended to suggest that this molecule was also an allosteric effector of the enzyme which promoted the 'fast' protein conformation [16]. In contrast, it is known from spectroscopic evidence that 'fast' and 'slow' oxidase preparations differ in the structure, and, in particular, the ligation state of the binuclear centre [2], and it has been shown by Bickar et al. [13,16] that a model in which a ligand bridging haem a_3 and Cu_B effectively lowers the electron affinity of the binuclear centre can adequately explain the intramolecular electron transfer characteristics of 'slow' oxidase, including the stimulatory effect of cytochrome *c*. The limitation to intramolecular electron transfer in this type of model is seen as being thermodynamic rather than kinetic. The 'ligation-state' model might also be applied to the 'open'/'closed' transition, where the very rapid cyanide-binding form could be seen as a particular state of the binuclear centre that occurs during turnover.

The work described in this paper stemmed from our desire to be able to interconvert the 'fast' and 'slow' forms of cytochrome-*c* oxidase in a controlled manner. It began with an investigation into the effects of low pH and formate on cytochrome oxidase, the former having been shown by Baker et al. [6] to accelerate the conversion of 'fast' to 'slow' oxidase, and the latter to induce the formation of the '$g' = 12$' EPR signal [17,18]. Initially, we paid particular attention to the changes in the cyanide-binding kinetics and the absorption spectrum in the Soret region produced by these treatments, and this led fortuitously to the novel observation that incubation of 'fast' oxidase with chloride or bromide at low pH produces forms of the enzyme which are similar with respect to their cyanide-binding properties to the 'slow' form of the enzyme. We have since also examined the changes in the dithionite-reduction kinetics and the EPR spectrum of 'fast' oxidase produced by these reagents. The aims of this paper are five-fold: (a) to present data that strengthen the notion that 'fast' and 'slow' oxidase are equivalent to 'pulsed' and 'resting' oxidase, respectively, (b) to show that ligation of 'fast' oxidase with formate produces a form of the enzyme that is difficult to distinguish from, but probably not identical with, the 'slow' form, (c) to show that neither chloride nor bromide can be the inhibitory ligand in 'slow' oxidase, even though they evidently

* When the pulsing procedure is carried out in this manner, usually using an excess of dithionite as the reductant, a major part of the initial product can be the 'peroxy' form of the enzyme which contains bound oxygen and two electrons more than the fully oxidised form [45]. This form then decays to the 'pulsed' form.

** For simplicity, in the remainder of this paper we shall use only the terms 'slow' and 'fast' when referring to the forms of cytochrome oxidase as prepared, i.e., we will assume that the 'slow' and 'resting' forms, and the 'fast' and 'pulsed' forms are equivalent, respectively. We have avoided using the term 'pulsed' because of possible confusion with the 'peroxy' and 'ferryl' forms of the enzyme which may be formed during pulsing procedures.

ligate the 'fast' enzyme and thereby produce changes in the rate of cyanide binding, (d) to demonstrate the presence of at least two subpopulations, which differ in reactivity to cyanide and formate, in 'fast' oxidase, and, finally, (e) to discuss the implications of these data with respect to possible protein conformational models versus ligand-binding/debinding models for the interconversion of the 'fast' and 'slow' forms of cytochrome oxidase.

Materials and Methods

Preparation of cytochrome-c oxidase

Cytochrome-*c* oxidase that mostly reacts rapidly with cyanide was prepared from bovine heart by various modifications of the Kuboyama et al. method [19]. The essential modification in all was the maintenance of high pH during the entire procedure but in particular during the preparation of the starting material (either a large-scale mitochondrial preparation (Prep. D) or a modified Keilin-Hartree preparation (Preps. A, B, C and E)).

The mitochondrial preparations were made essentially by the method of Smith (Procedure 3 using a Waring Blendor [20]) except that the medium was 50 mM sodium Bicine (pH 8.0), containing 250 mM sucrose and 1 mM potassium EDTA, and 2 M sodium Bicine (pH 9.0) was used instead of 2 M Tris base to adjust the pH to 8.0 at the blending stage. The Keilin-Hartree preparations were made essentially by the method of King [21] except that (1) the initial washing stages were omitted, (2) the heart meat was cubed and not minced before blending, and (3) blending was carried out in 50 mM sodium Bicine (pH 8.0) and 2 M sodium Bicine (pH 9.0) was used to adjust the pH to 8.0.

During the oxidase preparation sodium borate/phosphate (0.1 M each) (pH 8.5) was used as the medium. At the beginning the mitochondria were diluted to 20 mg mitochondrial protein/ml in this medium. In general, it was found that a satisfactory 'red/green' split was obtained from the mitochondrial preparations using about 30 μl/ml of 20% (w/v) cholate (compared to 40 μl/ml for Keilin-Hartree particles). The final precipitate was dissolved to about 400 μM haem A (200 μM enzyme 'monomer') in either 0.1 M potassium phosphate (pH 8.0) (Preps. A, B and D) or 0.1 M potassium Bicine (pH 8.5) (Preps. C and E), both containing 1 mM potassium EDTA and 0.1% Tween 80, and dialysed against the same buffer before storage in small lots in liquid nitrogen. All experiments, with the exception of the EPR measurements (Figs. 9 and 10), were carried out using enzyme that had been freshly thawed. For the EPR samples, enzyme that had been restored at $-20\,^{\circ}$C was used.

Optical measurements

Optical measurements were made with a single-beam spectrophotometer constructed round an $f/3.4$ monochromator (Applied Photophysics, Leatherhead, U.K.) fitted with a ruled grating (blazed at 300 nm). The detector was an end-window photomultiplier (9558QB, Thorn EMI Electron Tubes, Ruislip, U.K.) controlled via a model 7250 Photometric Control Unit (Applied Photophysics). A WG360 Schott glass high-pass filter was routinely used between the sample cuvette and the detector.

Cyanide-binding kinetics were monitored using a cycle of three measurements at 412, 432 and 412 nm. The difference in the absorbance at 432 nm and that at 412 nm was calculated using the average of the two measurements at 412 nm in each cycle, thereby achieving an essentially simultaneous dual-wavelength measurement. The cyanide-binding reaction was normally started by adding potassium cyanide solution (nominally 1 M, pH 7–7.5) to oxidase at 3–4 μM (final [cyanide], 32 mM). At this [cyanide] the slowest phase of binding examined had a half-time of 40–50 min, so it was not generally possible to obtain directly an accurate estimate of the maximal cyanide-binding signal. The maximal cyanide-binding signal for control oxidase was measured by adding the enzyme to 0.1 M potassium Mes buffer (pH 6.5), containing 1 mM potassium EDTA and 0.5 M potassium cyanide. At this [cyanide] the binding was almost complete after 20 min at $22\,^{\circ}$C. The end-point was then obtained by fitting two independent exponentials to the data using the simplex method [22]. A control measurement was also made, where enzyme was added to buffer that did not contain cyanide. As previously reported [4] the fastest phase of cyanide binding exhibited saturation behaviour since there was little increase in rate at 0.5 M cyanide when compared with 32 mM.

Dithionite-reduction kinetics were monitored in a manner similar to that used for the cyanide-binding kinetics but using a cycle of measurements at 462, 444 and 462 nm. The reaction was started by adding sodium dithionite solution (nominally 1 M, freshly made and kept under nitrogen) to a final concentration of 32 mM.

EPR measurements

Continuous-wave EPR measurements were recorded on a Bruker ESP300 spectrometer fitted with a TE103 rectangular cavity and an Oxford Instruments liquid helium flow cryostat (ESR900). All spectra were baseline corrected by subtraction of a spectrum of water obtained under identical conditions. A Hewlett Packard

microwave frequency counter 5350B was used for accurate calculations of g-values.

Results

Comments on the properties of the 'fast' cytochrome oxidase as prepared

Our 'fast' cytochrome oxidase as prepared is generally found to be partially electronated, i.e., some of the enzyme is in the 'peroxy' form and some is in the 'ferryl' form. To obtain a fully oxidised preparation it was necessary to preincubate the enzyme with catalase. For convenience this was generally added (1 μl/ml of 1:100 diluted Sigma C-100 suspension, approx. 40 U/ml final concentration) when the enzyme was redissolved before final dialysis. After removal of a batch of enzyme from storage in liquid nitrogen it was incubated at room temperature (about 20 °C) for 2–3 h before use. The enzyme preparations used in the experiments described in this paper were treated in this manner unless otherwise noted. After the catalase pretreatment the Soret maximum was found to be between 424 and 425 nm.

We have obtained preparations of oxidase where the contribution by the fast phase of cyanide binding is more than 95% of the total signal observed at 432–412 nm. However, such preparations are exceptional and in general the contribution by the fast phase is less (70–90%). The remaining signal is accounted for by a slow phase of cyanide binding with a rate constant about two orders of magnitude less than that of the fast phase. We have not as yet observed a phase with intermediate kinetics in our 'fast' enzyme as prepared.

The preparations (Preps. C and E, see Materials

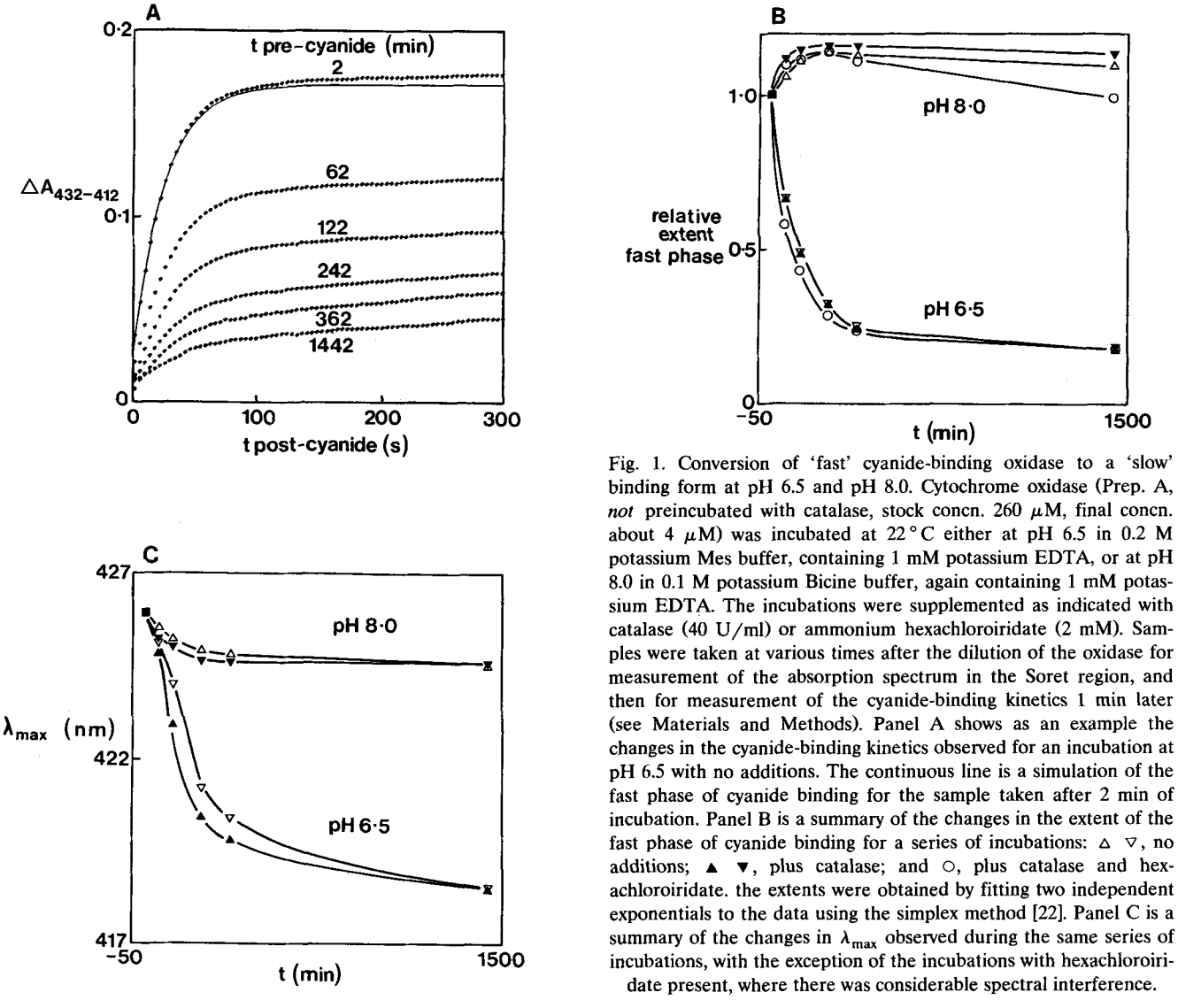

Fig. 1. Conversion of 'fast' cyanide-binding oxidase to a 'slow' binding form at pH 6.5 and pH 8.0. Cytochrome oxidase (Prep. A, *not* preincubated with catalase, stock concn. 260 μM, final concn. about 4 μM) was incubated at 22 °C either at pH 6.5 in 0.2 M potassium Mes buffer, containing 1 mM potassium EDTA, or at pH 8.0 in 0.1 M potassium Bicine buffer, again containing 1 mM potassium EDTA. The incubations were supplemented as indicated with catalase (40 U/ml) or ammonium hexachloroiridate (2 mM). Samples were taken at various times after the dilution of the oxidase for measurement of the absorption spectrum in the Soret region, and then for measurement of the cyanide-binding kinetics 1 min later (see Materials and Methods). Panel A shows as an example the changes in the cyanide-binding kinetics observed for an incubation at pH 6.5 with no additions. The continuous line is a simulation of the fast phase of cyanide binding for the sample taken after 2 min of incubation. Panel B is a summary of the changes in the extent of the fast phase of cyanide binding for a series of incubations: △ ▽, no additions; ▲ ▼, plus catalase; and ○, plus catalase and hexachloroiridate. the extents were obtained by fitting two independent exponentials to the data using the simplex method [22]. Panel C is a summary of the changes in λ_{max} observed during the same series of incubations, with the exception of the incubations with hexachloroiridate present, where there was considerable spectral interference.

and Methods) used to obtain the results in Figs. 4 and 11 were produced by a slightly modified procedure. The modification consisted of including 30 mM sodium ascorbate and 0.5 mM *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine in the medium during the first solubilisation of the Keilin-Hartree particles with cholate, and thereafter including 40 U/ml of catalase. The aim of this was to produce preparations which were certain to show no slow phase of cyanide binding.

The effect of pH on the cyanide-binding kinetics and optical spectra of 'fast' cytochrome oxidase

Fig. 1 shows the results of an experiment in which 'fast' oxidase (Prep. A) was incubated at pH 6.5 or 8.0 in the presence of catalase, or catalase and hexachloroiridate, or in the absence of any additions. Panel A shows the time-courses of cyanide binding to samples of the enzyme taken at various times from the pH 6.5 incubation with no additions, as monitored using the wavelength pair 432–412 nm; panel B summarises the changes in the extent of the fast phase of cyanide binding obtained from such time-courses, and panel C shows the changes in the position of λ_{max} in the Soret region observed during each incubation. There are several points of interest in these results.

(a) The basic findings of Baker et al. [6] are confirmed, since over 24 h at low pH there is a blue shift in the position of λ_{max} in the Soret region from 426 nm to 418.5 nm (Fig. 1, panel C, ▽ or ▲) with a concomitant decrease in the extent of the fast phase of cyanide binding to about 20% of its initial value (Fig. 1, panels A and B, ▽ or ▲).

(b) The presence of partially-electronated forms of oxidase in this preparation as noted in the previous section and their disappearance with time is clear, since incubation of the enzyme at high pH, with or without catalase, leads to a small blue shift in λ_{max} to 424.5 nm (Fig. 1, panel C, △ and ▼, respectively) accompanied by an *increase* in the fast phase of cyanide binding (Fig. 1, panels B, △ and ▼, respectively). The explanation for this behaviour lies in the similarity of the absorption spectra in the Soret region of cyanide-ligated oxidase and the 'peroxy' and 'ferryl' forms of the enzyme. Consequently, we would expect to observe virtually no binding signal at 432–412 nm when cyanide reacts with the 'peroxy' and 'ferryl' forms. In any case, cyanide may bind only slowly to the 'peroxy' and 'ferryl' forms of the enzyme.

(c) The effect of the oxidant hexachloroiridate, which accelerated the conversion of the 'fast' form to the 'slow' form at both pH values (Fig. 1, panel B,○), provides a possible explanation for at least some of the variability noted previously by Baker et al. [6] in the stability of 'fast' oxidase, particularly at pH 8.0. We suggest that slow 'leaks' of electrons from the environment serve to pulse some of the enzyme molecules and thereby lower the apparent 'fast' to 'slow' conversion rate. In connection with this, we have observed the spontaneous generation of the 'peroxy' and 'ferryl' forms of the enzyme on incubation of 'fast' oxidase at pH 8.0. The addition of EDTA was found to lessen this.

(d) A few of our oxidase preparations (e.g., Prep. B, Figs. 2, 3, 5–8) appear to reach a steady state mixture of 'fast' and 'slow' forms on incubation at pH 6.5, i.e., the rate of conversion of 'fast' to 'slow' matches the rate of reactivation caused by slow turnover of the enzyme. In such cases the steady state level of the fast phase of cyanide binding is lowered by the addition of hexachloroiridate. However, there is always a fraction of the fast cyanide-binding phase which remains on incubation at pH 6.5 even in the presence of a sink for electrons such as hexachloroiridate.

The effects of formate, chloride and bromide on the cyanide-binding kinetics and optical spectra of cytochrome oxidase

Fig. 2 shows the results of similar experiments to those in Fig. 1 in which 'fast' oxidase (Prep. B) was incubated at pH 6.5 in the presence of either a low concentration of formate (50 μM) or a high concentration of chloride or bromide (0.5 M). It can be clearly seen that the inclusion of any of these anions produces progressive changes in the kinetics of cyanide binding. In the case of formate and bromide the fast phase of binding decreases in extent, while the slow phase increases in extent (panels A and B, respectively). In the case of chloride the fast phase decreases in extent, while there is a corresponding increase in a phase with intermediate kinetics (rate constant = 0.0057 ± 0.0004 s^{-1}, S.E.M., from fits to the time-courses in panel C, with the exception of that after 2 min of incubation). The results of control experiments in which samples of Prep. B oxidase were incubated at pH 6.5 in the absence of additions or in the presence of 0.25 M potassium sulphate (not shown) show only a small decrease in the extent of the fast phase of cyanide binding over the same period of time (Fig. 2, panel D, ●).

Fig. 3 shows examples of the spectral changes in the Soret region induced by incubation with chloride, bromide and formate. All three anions induce a blue shift in the Soret maximum. The formate-induced and the bromide-induced changes are similar, although the latter is of much smaller amplitude, and the difference spectra for these changes are like that obtained on incubation of 'fast' oxidase at pH 6.5 (not shown). However, the chloride-induced difference spectrum, although of similar amplitude to that induced by bromide is noticeably different, consisting largely of a simple loss of absorbance centred at about 430 nm. The difference spectra of the blue shift induced by

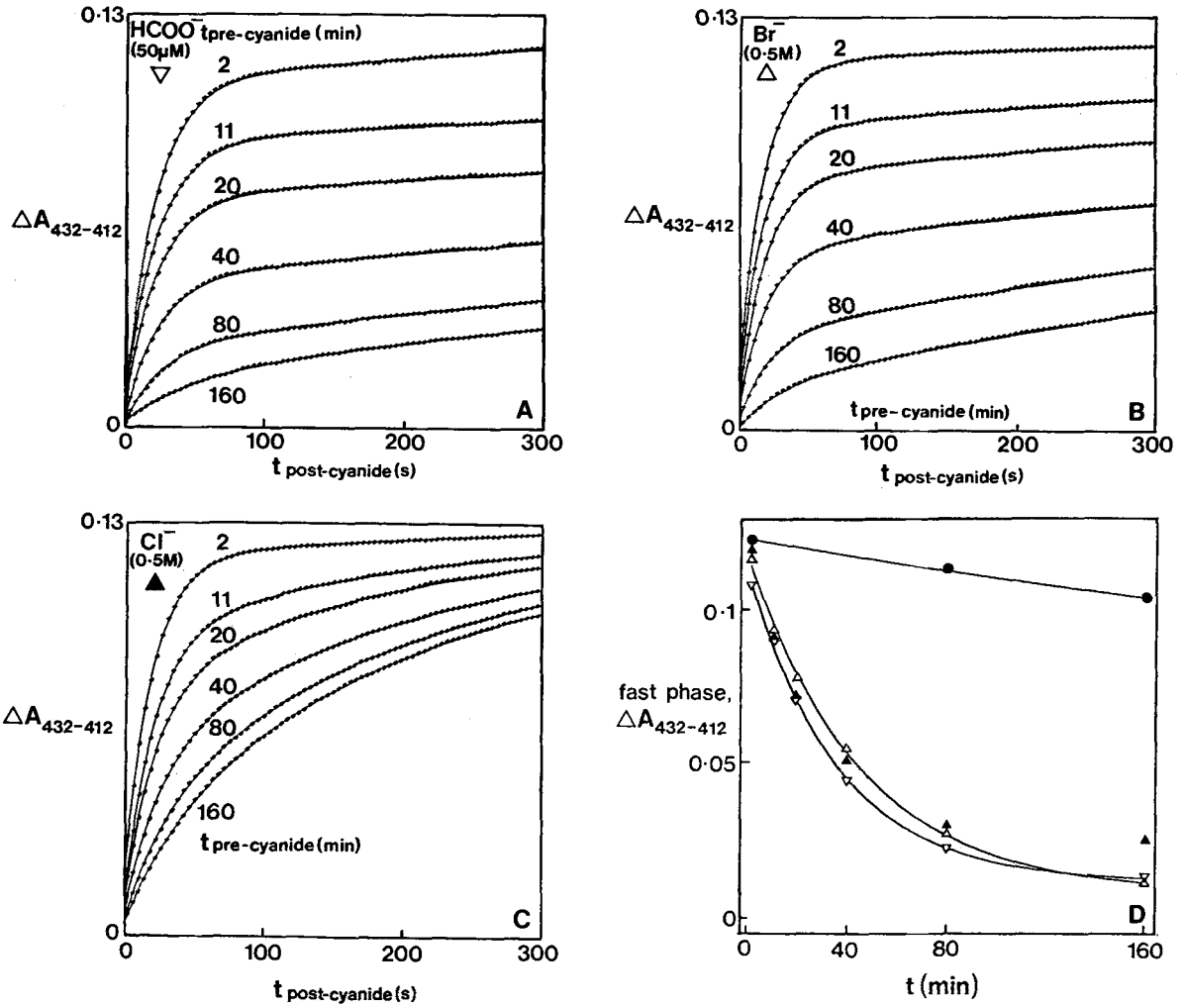

Fig. 2. Conversion of 'fast' cyanide-binding oxidase to 'slow' binding forms at pH 6.5 by formate, chloride and bromide. Cytochrome oxidase (Prep. B, preincubated with catalase, stock concn. 180 μM, final concn. about 3 μM) was incubated at 22 °C at pH 6.5 in 0.2 M potassium Mes buffer, containing 1 mM potassium EDTA. The incubations were supplemented as indicated with potassium formate (50 μM), potassium chloride (0.5 M) or potassium bromide (0.5 M). Samples were taken at various times after the dilution of the oxidase for measurement of the absorption spectrum in the Soret region, and then for measurement of the cyanide-binding kinetics one minute later (see Materials and Methods section). Panels A, B and C show the changes in the cyanide-binding kinetics observed for incubation with formate, bromide and chloride, respectively. The solid lines in each case are simulations. The parameters for these simulations were obtained by fitting two independent exponentials (panels A and B) or three independent exponentials (panel C) to the data using the simplex method [22]. Panel D is a summary of the changes observed during these incubations in the extent of the fastest phase of cyanide binding: ●, no additions; ▽, formate; △, bromide; and ▲, chloride. The continuous lines were obtained by fitting single exponentials to the formate and bromide data. The rate constants for these exponentials were found to be 0.028 min^{-1} and 0.023 min^{-1}, respectively.

formate shown in Fig. 3 (panel A) are consistent with a change in the electronic configuration of haem a_3 from low-spin to high-spin, as proposed by Nicholls [23], i.e., it is essentially the inverse of the cyanide-binding spectrum in this region (see, for example, Ref. 4). If this assignment is correct, then there must be an equilibrium mixture of the low-spin and high-spin forms of haem a_3 in 'fast' oxidase at room temperature, and so we would then expect the extent of the total cyanide-binding signal to increase during incubations with formate (or bromide) as the fraction of low-spin haem a_3 already present decreases. This is indeed found to be the case (data not shown).

Heterogeneity in our 'fast' oxidase preparations

An interesting feature of the results shown in Figs. 1 and 2 is that in all cases there is a fraction of the fast cyanide-binding phase that remains even on prolonged incubation, indicative of heterogeneity in the 'fast' subpopulation of our oxidase. There are two other observations which support this point of view. (a) The rate constant for the residual fast phase of cyanide binding is found to be significantly lower than that observed at the start of the incubations (Table I). (b) On incubation with a high concentration of formate (5 mM) the changes induced in cyanide-binding kinetics are biphasic. The main body of the fast cyanide binding

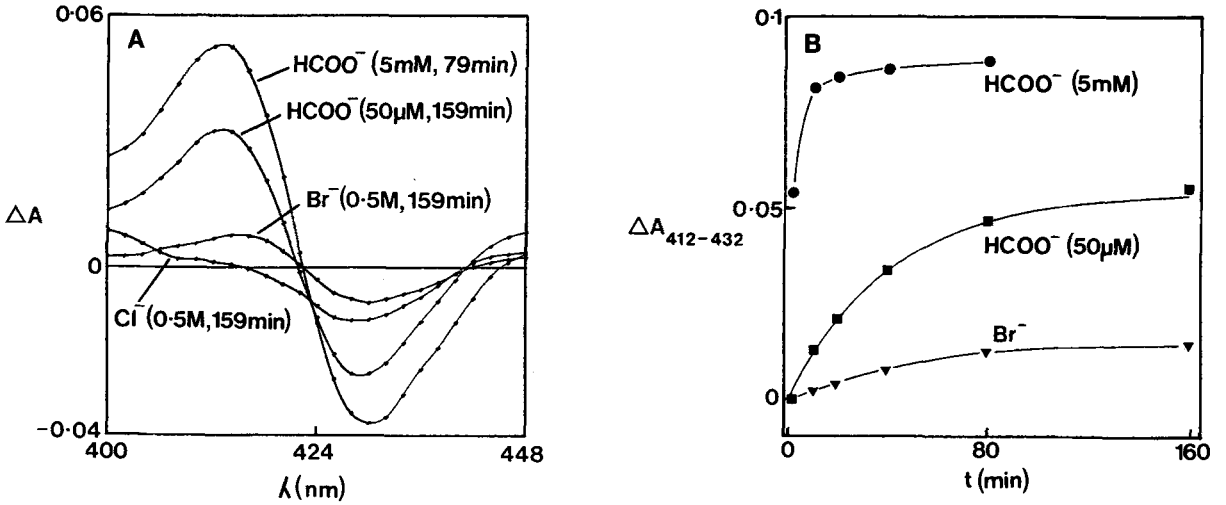

Fig. 3. Conversion of 'fast' cyanide-binding oxidase to 'slow' binding forms at pH 6.5 by formate, chloride and bromide: Spectral changes in the Soret region. The data shown were obtained from the same set of incubations as the data shown in Fig. 2 (see legend of that figure for details). Panel A shows kinetic difference spectra. For the incubations with 0.5 M bromide and 50 μM formate these spectra were obtained by subtracting the spectrum obtained after 1 min of incubation from the spectrum obtained at the time indicated. The initial rate of change for the incubation with 5 mM formate was very rapid and so in this case the spectrum was obtained by subtracting the spectrum after 1 min incubation in the presence of 50 μM formate. Panel B shows the time courses of $\Delta A_{412-432}$ obtained in the same way. The continuous line was obtained by fitting a single exponential to the low [formate] data. The rate constant for this exponential was found to be 0.025 min^{-1}.

phase disappears within 2 min accompanied by a rapid spectral change. The residual fast phase then disappears (within about 20 min) in association with a further spectral change, similar to the first phase, and that observed with the low concentration of formate (50 μM), but of relatively large amplitude in comparison to the change in the extent of the fast phase of cyanide binding associated with it (Fig. 2, panel D, and Fig. 3).

These observations are consistent with there being a subpopulation of 'fast' cyanide binding oxidase molecules in which the equilibrium between the high-spin and low-spin forms of haem a_3 is shifted further towards the low-spin form and which binds formate more slowly than the remaining 'fast' cyanide binding subpopulation.

TABLE I

The effect of various treatments on the pseudo-first-order rate constant for the fast phase of cyanide binding to cytochrome oxidase

Experimental details were as described in the legends to Figs. 1 and 2.

Preparation	pH	Additions	k', fast phase (s^{-1})	
A	6.5		2 min	24 h
		none	0.040	0.027
		catalase	0.037	0.028
		catalase & hexachloroiridate	0.043	0.026
A	8.0		2 min	24 h
		none	0.029	0.027
		catalase	0.029	0.031
		catalase & hexachloroiridate	0.029	0.029
B	6.5		2 min	24 h
		none	0.043	0.036
		sulphate (0.25 M)	0.041	0.036
B	6.5		2 min	160 min
		formate (50 μM)	0.044	0.019
		bromide (0.5 M)	0.054	0.030
		chloride (0.5 M)	0.049	0.024

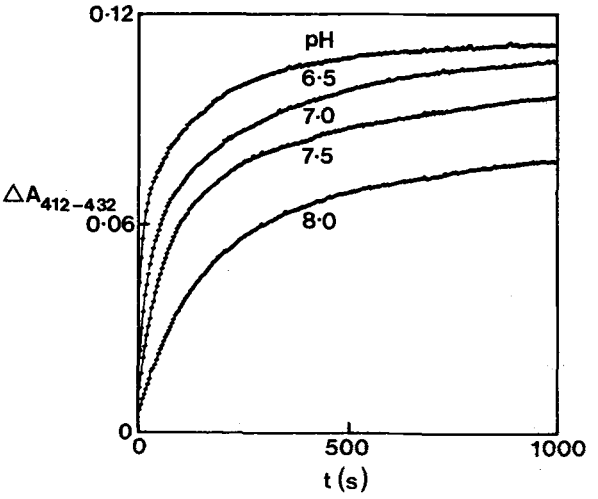

Fig. 4. Effect of pH on the formate-binding kinetics of 'fast' oxidase. In each case the binding was initiated at 20 °C by adding formate (to a final concn. of 32 mM) to 0.1 M Bistris propane sulphate buffer (pH as indicated), containing 1 mM potassium EDTA and 3 μM cytochrome oxidase (Prep. C, preincubated with catalase, stock concn. 190 μM).

The pH-dependence of the effects of formate, chloride and bromide

The biphasic effect of formate on 'fast' oxidase is shown more clearly in Fig. 4, where the reaction with formate was monitored at different pH values using the wavelength pair 412–432 nm. At pH 6.5 a fast phase of response is observed with a rate constant of about 0.07 s^{-1}; the slow phase, which accounts for about 42% of the total signal, has a rate constant about an order of magnitude less. Both phases are slower at pH 8.0 (0.18 and 0.23 of the rates at pH 6.5, respectively). The contributions by the two phases are found to be remarkably constant from preparation to preparation, for seven preparations (with Soret maxima > 424 nm) the extents of the fast and slow phases were 0.125 ± 0.004 (S.E.) and 0.099 ± 0.005, respectively, relative to A_{422}.

The reactions with chloride and bromide have pH dependencies similar to those of the reaction with formate in that no effect on the cyanide-binding kinetics of 'fast' oxidase can be discerned when the enzyme is incubated at pH 8.0 with either anion at 0.5 M. This implies that the pH dependence of the formate effect might arise from the (de)protonation of a group on the protein, and weakens the suggestion that HCOOH is the binding species [23].

Once the reaction of oxidase with chloride has taken place the resulting intermediate phase of cyanide binding also shows pH dependence. This, together with the pH-dependence of the rate of reaction of chloride with oxidase, is illustrated in Fig. 5 and Table II. The former shows the change in the extent of the fast phase of cyanide binding to 'fast' oxidase after incubation for 1 h at 22 °C in the presence of 0.5 M KCl at pH 6.0, 6.5 or 7.0. The latter shows the fitted rate constants and extents for the fast and intermediate phases. It can be seen (a) that the fast phase decreases more after 1 h incubation at pH 6.0 than after 1 h incubation at pH 7.0, and (b) that the rate constant for the fast phase decreases over the pH range 6–7 (see also Tables I and III for the difference in this value between pH 6.5 and 8.0), whereas the rate constant for the intermediate phase *increases* over this pH range. In all cases the increase in the extent of the intermediate phase of cyanide-binding corresponds, within experimental error, to the decrease in the fast phase.

Dithionite reduction

We have also looked at the effects of chloride, bromide and formate on the dithionite reduction kinetics. Fig. 6 shows the results for incubations carried out in exactly the same way as those in Fig. 2. The absorbance change at the wavelength pair 444–462 nm was used to monitor the reduction of the enzyme by dithionite. It is generally agreed that both haems absorb to about the same extent at 444 nm and that 462 nm is approximately isosbestic for both haems [24–28].

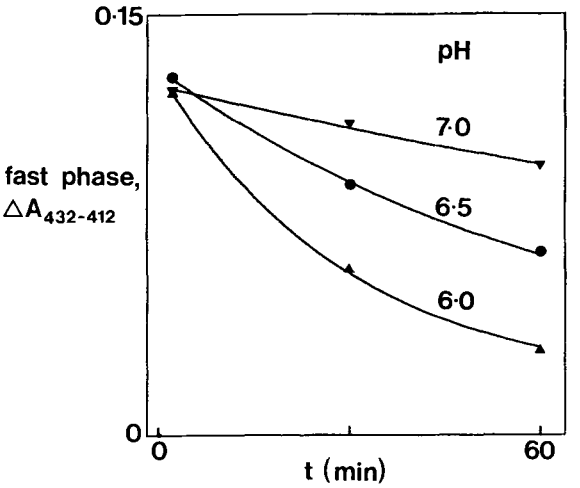

Fig. 5. Effect of pH on the chloride-binding kinetics of 'fast' oxidase. Cytochrome oxidase (Prep. B, preincubated with catalase, stock concn. 180 μM, final concn. about 3 μM) was incubated at 22 °C in 0.1 M Bistris propane sulphate buffer, containing 1 mM potassium EDTA and 0.5 M KCl, at pH values 6.0, 6.5 and 7.0, as indicated. At various times after the dilution of the oxidase samples were taken, first for measurement of the absorption spectrum in the Soret region, and then for measurement of the cyanide-binding kinetics 1 min later (see Materials and Methods section). The figure is a summary of the changes observed during these incubations in the extent of the fastest phase of cyanide binding. The continuous lines are single exponential decays to $\Delta A_{432-412} = 0.016$. The rate constants for these are 0.033 min^{-1}, 0.014 min^{-1} and 0.005 min^{-1}, for pH values 6.0, 6.5 and 7.0, respectively.

Some care needs to be taken when analysing the kinetics of dithionite reduction, since we have mixtures of 'fast' and 'slow' oxidase molecules. For 'fast' oxidase the reduction of haem a and the reduction of haem a_3 are kinetically indistinguishable under the conditions used here (a clear isosbestic point is observed at 433 nm.). For 'slow' oxidase the reduction of the two haems is clearly distinguishable: haem *a* is rapidly reduced (isosbestic at 438 nm) followed by haem a_3 at a much slower rate (isosbestic at 428 nm) (see, for example, Ref. 13). For simplicity, we have analysed our data by

TABLE II

The effect of pH on the reaction of chloride with 'fast' cytochrome oxidase, and on the rate of cyanide binding to chloride-ligated oxidase

Experimental details were as described in the legend to Fig. 5.

pH	Incubation time (min)	Fast		Intermediate	
		k' (s^{-1})	extent $\Delta A_{432-412}$	k' (s^{-1})	extent $\Delta A_{432-412}$
6.0	2	0.054	0.122		
	60	0.041	0.030	0.0048	0.088
6.5	2	0.050	0.127		
	60	0.040	0.065	0.0064	0.066
7.0	2	0.042	0.123		
	60	0.042	0.096	0.0103	0.034

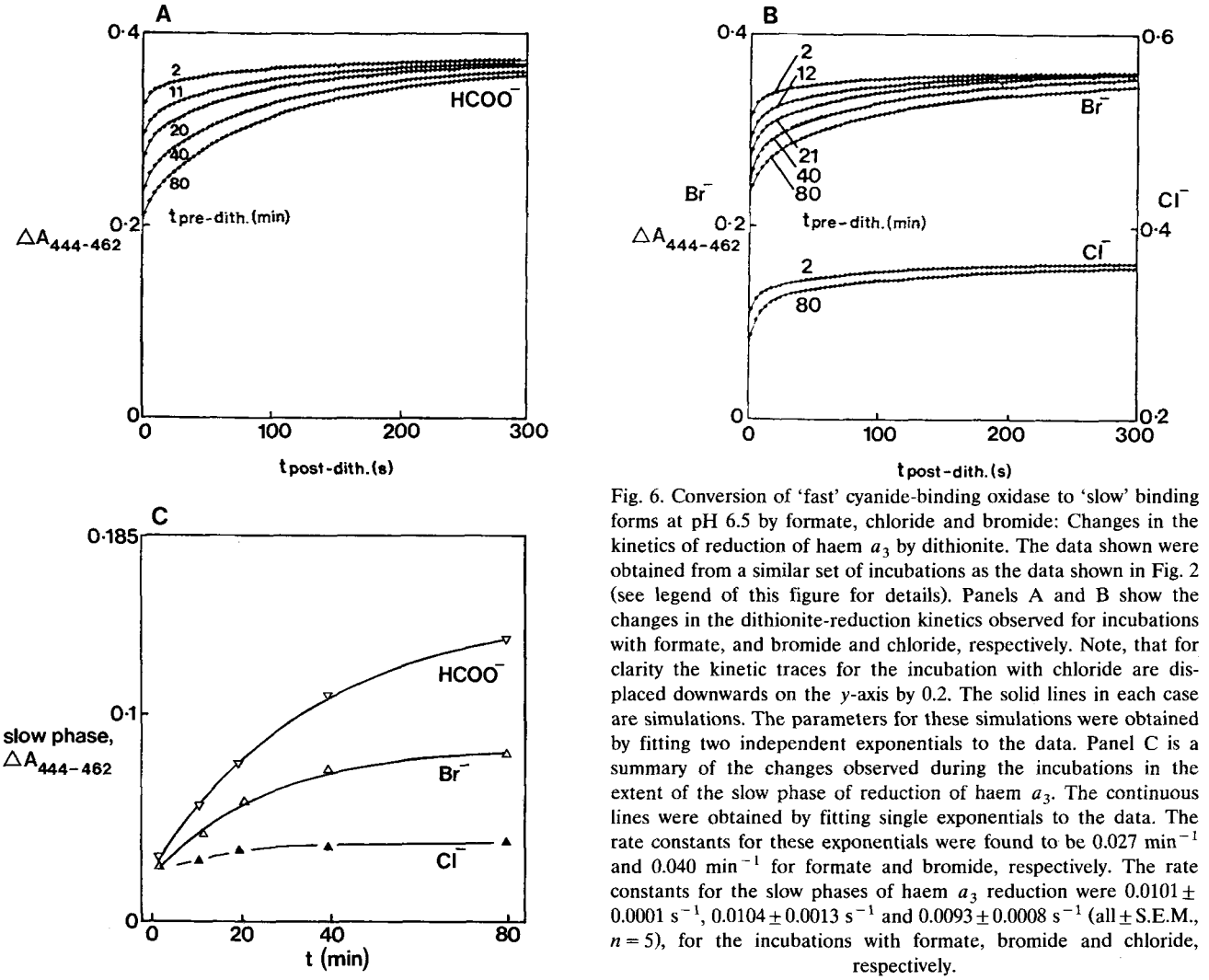

Fig. 6. Conversion of 'fast' cyanide-binding oxidase to 'slow' binding forms at pH 6.5 by formate, chloride and bromide: Changes in the kinetics of reduction of haem a_3 by dithionite. The data shown were obtained from a similar set of incubations as the data shown in Fig. 2 (see legend of this figure for details). Panels A and B show the changes in the dithionite-reduction kinetics observed for incubations with formate, and bromide and chloride, respectively. Note, that for clarity the kinetic traces for the incubation with chloride are displaced downwards on the y-axis by 0.2. The solid lines in each case are simulations. The parameters for these simulations were obtained by fitting two independent exponentials to the data. Panel C is a summary of the changes observed during the incubations in the extent of the slow phase of reduction of haem a_3. The continuous lines were obtained by fitting single exponentials to the data. The rate constants for these exponentials were found to be 0.027 min^{-1} and 0.040 min^{-1} for formate and bromide, respectively. The rate constants for the slow phases of haem a_3 reduction were 0.0101 ± 0.0001 s^{-1}, 0.0104 ± 0.0013 s^{-1} and 0.0093 ± 0.0008 s^{-1} (all $\pm$ S.E.M., $n = 5$), for the incubations with formate, bromide and chloride, respectively.

fitting two independent exponential processes. However, it is only the second of these, which represents the slow intramolecular reduction of haem a_3 in 'slow' oxidase molecules, that we regard as having any meaning.

On incubation with 50 μM formate the extent of the slow phase of haem a_3 reduction increases (panels A and C, ▽) at a rate similar to that observed under identical conditions for the decrease in the extent of the fast phase of cyanide binding and for the blue shift in the Soret peak (Fig. 2, panel D, ▽ and Fig. 3, panel B, ■ *). Throughout the incubation the final signal reached on completion of the reduction by dithionite (obtained by curve fitting) stays constant within experimental error. Incubation with 0.5 M bromide has a similar effect (panels B and panels C, △) but the increase is smaller. Incubation with 0.5 M chloride had virtually no effect on the dithionite-reduction kinetics. For both bromide and chloride, as with formate, the final signal on dithionite reduction stays constant within experimental error.

Again, we see evidence for the heterogeneity in the reactivity of the 'fast' subpopulation of the enzyme with formate. If we estimate the final extent of the slow phase by fitting an exponential to the increase in this phase of dithionite reduction produced by incubation with 50 μM formate we find a considerable shortfall between this value (0.150) and that expected if all the haem a_3 were reduced in this phase (0.185, i.e., the maximal extent of $\Delta A_{444-462}$, 0.369, divided by 2). This is consistent with there being two phases in the reac-

* Some variation was noted in the amplitude of the spectral change induced by incubation with 0.5 M bromide. In the experiment shown in Fig. 6, $\Delta A_{412-432}$ was found to be 0.020 after 79 min, compared to 0.012 after 79 min found in the experiment shown in Fig. 3.

tion with formate as noted before (see Fig. 4). Hence, there must be at least three subpopulations of oxidase molecules in our 'fast' preparations: –

(1) A small and variable subpopulation of 'slow' oxidase molecules which bind cyanide slowly, in which the reduction of haem a_3 by dithionite is slow, and in which haem a_3 is in the high-spin form.

(2) A majority subpopulation of 'fast' oxidase molecules which bind cyanide about 100-fold more rapidly than the 'slow' molecules; which react fairly rapidly with formate, in which the reduction of haem a_3 by dithionite is rapid, and in which some of the haem a_3 is in the low-spin form.

(3) A minority subpopulation of 'fast' oxidase molecules that, like those in subpopulation 2, bind cyanide rapidly and show rapid reduction of haem a_3 by dithionite, but which differ from those in subpopulation 2 in that they react with cyanide at a slightly lower rate, with formate at a much lower rate, and have haem a_3 that is mostly in the low-spin form.

We can verify that our results are self-consistent on this point by plotting together the normalised time-courses for the development of the slow phase of reduction of haem a_3 by dithionite and for the development of the slow phase of cyanide binding, both in the presence of 50 μM formate (Fig. 7). It can be seen that the two time-courses agree reasonably well. By fitting two independent exponentials to each of these time-courses we have obtained values of 0.26 and 0.31, respectively, for the fraction of the slow formate binding subpopulation 3.

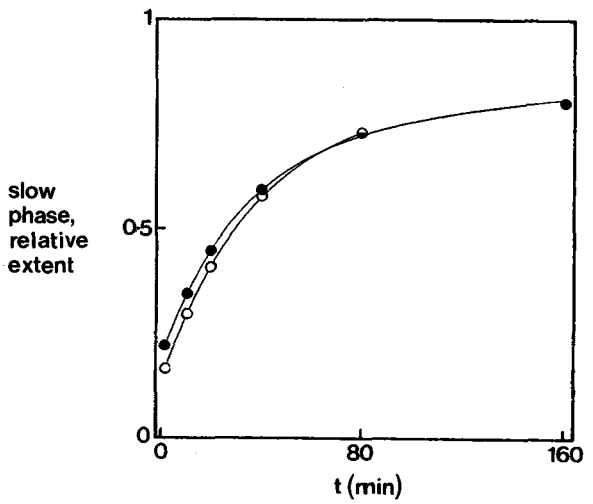

Fig. 7. Time-courses for the development of the slow phase of cyanide binding (●) and the slow phase of dithionite reduction (○) during incubations of 'fast' cytochrome oxidase with 50 μM formate. This is a replot of data from Figs. 2 and 6. The extent of the slow phase of cyanide binding was estimated by adding the extent of the cyanide-binding signal observed for control enzyme obtained using 0.5 M cyanide ($\Delta A_{432-412} = 0.166$, see Materials and Methods) to the extent of the formate-binding signal observed at a given time. The maximal extent of the formate-binding signal ($\Delta A_{412-432} = 0.088$) was obtained using 5 mM formate. The continuous curves were obtained by fitting two independent exponential processes, with the rate constant for the slow phase constrained to 0.093-times the rate constant of the fast phase, in accord with formate-binding kinetic data from seven oxidase preparations. The rate constants for these fits were 0.029 min^{-1} and 0.033 min^{-1} for the dithionite-reduction data and the cyanide-binding data, respectively.

Evidence for the retention of the effects of chloride, bromide and formate after dialysis

In the experiments described so far, the kinetics of cyanide binding were measured in the presence of essentially the same concentrations of formate, chloride and bromide as were present in the preincubations. Therefore, it is not clear whether their effects on the cyanide binding kinetics of oxidase require their continued presence. To test this we incubated 'fast' oxidase at relatively high concentration at pH 6.4 with chloride, bromide or formate, and then dialysed the enzyme samples thoroughly at pH 8.0. The cyanide-binding kinetics assayed at pH 8.0 before and after dialysis for the bromide and formate incubations, and a control incubation with no additions are shown in Fig. 8, panels C, D and A, respectively. panel B shows a comparison of the cyanide-binding kinetics for the chloride incubation assayed after dialysis at pH 6.5 as well as pH 8.0. Table III is a summary of the fitted rate constants and the extents of the fast phases of cyanide binding obtained by fitting two (or three) independent exponentials to the kinetic data. The position of the Soret maximum of each sample is also shown, and where appropriate the rate constant and extent of the intermediate phase of cyanide binding. The dialysed samples were slightly more dilute than those from the original incubations so the kinetics shown were scaled according to the ratio of the A_{422} before dialysis to the A_{422} after dialysis. This point is close to being isosbestic for any spectral changes observed during the incubations (see, for example, Fig. 3).

In the control incubation the decrease in the extent of the fast phase assayed at pH 8.0 was as expected for 1 h incubation at 22 °C and pH 6.5 (cf. control values, assayed at pH 6.5, in Fig. 2, panel D, Fig. 5 and Table II, where the same preparation of oxidase was used). However, the further large decrease during the dialysis at pH 8.0 was somewhat unexpected (cf. Fig. 1, panel B). As noted earlier, the oxidase preparation used here appeared to contain a steady-state level of the 'slow' form after incubation for 24 h at pH 6.5 and 22 °C, where the rate of reversion of 'fast' to 'slow' matched the rate of reactivation caused by slow turnover of the enzyme by electrons 'leaking' from the environment. It may be that the temperature is an important factor in determining the relative rates of these two processes, since the dialysis was carried out at 4 °C.

The results with chloride are interesting. It is not possible to resolve the intermediate phase of cyanide

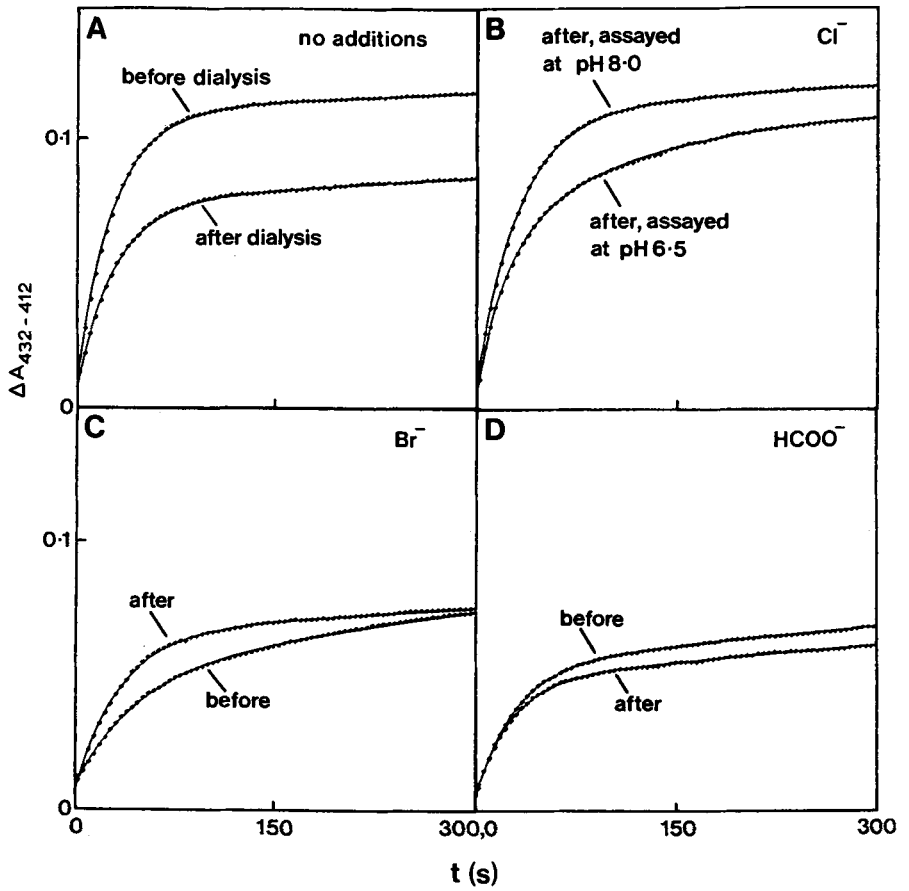

Fig. 8. Effect of dialysis on the effects of chloride, bromide and formate on 'fast' oxidase. Four incubations were set up at pH 6.4 by mixing 200 μl of oxidase (approx. 180 μM, Prep. B) at pH 8.0 in 0.1 M K-P_i buffer, containing 1 mM potassium EDTA and 0.1% Tween 80, with 400 μl of 0.2 M potassium Mes buffer (pH 6.0) containing 1 mM potassium EDTA and one of the following: (A) no additions; (B) 0.75 M KCl; (C) 0.75 M KBr; on (D) 75 μM potassium formate. After 1 h at 22 °C a sample was taken from each incubation and used to assay the cyanide-binding kinetics at pH 8.0 (in 0.1 M potassium Bicine buffer, containing 1 mM potassium-EDTA). The remainder of each was dialysed at 4 °C against pH 8.0 buffer (three changes of 100 ml each per incubation) over 24 h after which further samples were taken for assay of the cyanide-binding kinetics. Panels are self-explanatory.

binding at an assay pH of 8.0 because of the marked pH-dependency of the cyanide-binding kinetics of the chloride-ligated enzyme. However, after incubation for 1 h at pH 6.4 and 22 °C some protection by chloride against the reversion of the 'fast' oxidase to the 'slow' form is apparent (Table III, see also Table II), and this

TABLE III

The effect of dialysis on the cyanide-binding kinetics of cytochrome oxidase treated with chloride, bromide and formate

The experimental details are given in the legend to Fig. 7.

Addition	Dialysis	Fast		Intermediate		Soret maximum (nm)
		k' (s^{-1})	extent $\Delta A_{432-412}$	k' (s^{-1})	extent $\Delta A_{432-412}$	
None	before	0.036	0.111			423.8
	after	0.035	0.077			421.8
Formate (50 μM)	before	0.034	0.054			419.9
	after	0.037	0.049			420.4
Bromide (0.5 M)	before	0.030	0.038	0.005	0.047	420.4
	after	0.029	0.067			421.3
Chloride (0.5 M)	before	0.022	0.117			421.8
	after (pH 8.0)	0.031	0.112			423.8
	after (pH 6.5)	0.045	0.059	0.008	0.055	

protection seems to be maintained during the dialysis at pH 8.0, since there is only a small decrease in the extent of the fast phase. In addition, when the cyanide-binding kinetics of the dialysed oxidase are assayed at pH 6.5, a phase with intermediate cyanide-binding kinetics characteristic of the chloride-ligated enzyme can clearly be seen, indicating that most of the effect of chloride is retained after dialysis.

Incubation with 0.5 M bromide at pH 6.5 leads to a decrease in the extent of the fast phase. It should be noted that in this experiment the [bromide] in the assay before dialysis was only 23 mM compared to 0.5 M in the experiment shown in Fig. 2. However, despite this, the decrease after 60 min is as expected from the data shown in Fig. 2 (panel D). A phase of cyanide binding with intermediate kinetics is clearly visible when the cyanide-binding kinetics are assayed at pH 8.0 (Fig. 8, panel C). Therefore, it seems probable that bromide-ligated oxidase shows a pH-dependent reaction with cyanide, like the chloride-ligated enzyme. Unlike the incubation with chloride, the extent of the intermediate phase does not correspond to the decrease in the fast phase compared to that observed in control enzyme. After dialysis, the intermediate phase is absent and there is some recovery of the fast phase. It would appear, therefore, that the effect of bromide has been lost. Nevertheless, it is clear that the recovery of the fast phase still falls short of the extent observed in the control incubation.

As expected, incubation with 50 μM formate at pH 6.5 leads to a considerable decrease in the extent of the fast phase of cyanide binding, and it is clear that dialysis does not lead to a recovery of this phase. Note, that in this experiment the formate was slightly substoichiometric with respect to the oxidase (approx. 60 μM), and so pseudo-first-order kinetics for the binding of formate no longer apply.

EPR spectra

Fig. 9 shows the effects of formate, chloride and bromide on the low-field (haem) EPR spectra of 'fast' cytochrome oxidase. A sample of a control preparation (Prep. D) of 'fast' enzyme at pH 8.0 (Fig. 9a) has an essentially identical spectrum to that seen in 'pulsed'

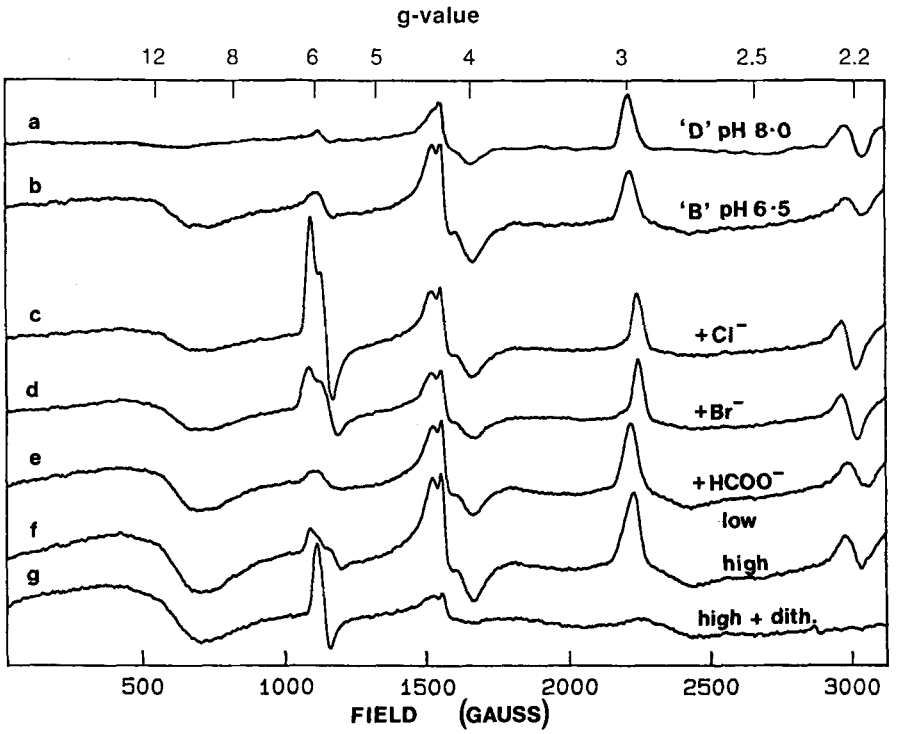

Fig. 9. The effect of chloride, bromide and formate on the haem EPR signals of 'fast' cytochrome oxidase. Sample (a) contained 51 μM oxidase (Prep. D) incubated at 24 °C for 1 h in 0.1 M potassium Bicine buffer (pH 8.0) containing 1 mM potassium EDTA. Samples (b)–(f) contained 60 μM oxidase (Prep. B, rethawed) incubated at 24 °C for 1 h in 0.2 M potassium Mes buffer (pH 6.4), containing 1 mM potassium EDTA and one of the following: (b) no addition; (c) 0.5 M KCl; (d) 0.5 M KBr; (e) 50 μM potassium formate; and (f) 5 mM potassium formate. Sample (g) was the same as sample (f) except that after 1 h incubation 20 mM buffered sodium dithionite (pH 6.5) was added and the EPR tube was frozen about 10 s later in liquid methanol cooled in a liquid nitrogen bath. The EPR conditions were as follows: temperature, 10 K; microwave power, 20 mW; microwave frequency, 9.35 GHz; modulation frequency, 100 kHz; modulation amplitude, 10.1 gauss; gain, 1.25E+5; sweep rate, 18.5 gauss/s; time constant, 328 ms; and conversion time, 82 ms. Each trace is the average of three scans. To allow for small variations in tube diameters, the spectral intensity of the samples was normalised against the double integral of their Cu_A EPR signal (see Fig. 10) at 70 K. Note that these conditions were chosen to facilitate comparison between all the different EPR signals. As a consequence the haem *a* signals at $g = 3$ and $g = 2.2$ are partly saturated with microwave power.

cytochrome oxidase [29] and the 'fast' enzyme prepared by Baker et al. [6]. There are haem *a* signals at $g = 3$ and 2.2, and only a very small amount of the '$g' = 12$' signal characteristic of 'slow' enzyme [6]. Also present are a $g = 4.3$ rhombic non-haem iron signal and a very small $g = 6$ high spin haem signal. These two signals represent only small amounts of the total iron present in the enzyme preparation and vary somewhat from preparation to preparation.

Incubation of 'fast' oxidase (Prep. B) at pH 6.5 increases the amplitude of the '$g' = 12$' signal (Fig. 9b). The addition of chloride to the incubation medium has little further effect on this signal (c), although an increase in amplitude is seen in the presence of bromide (d). However, the addition of formate produces a larger increase in the amplitude of the '$g' = 12$' signal (e), and, when a high concentration is used (5 mM, f) the signal produced is equivalent to the size of the signal seen in 'slow' enzyme [2].

The addition of chloride, and to a lesser extent bromide, causes a significant increase in the $g = 6$ signal. However, quantitation of this signal with respect to a metmyoglobin standard shows that chloride only induces this signal in at most 4% of the enzyme molecules (Table IV).

Some effects on the haem a EPR signals induced by the inclusion of chloride or bromide, and, to a lesser extent, the higher concentration of formate, should be noted. The highest *g*-value is decreased, whereas the other two are increased (Table IV). Therefore, as expected, there is no significant effect on the sum of the squares of the three *g*-values [30]. Similar

TABLE IV

The effect of incubation with chloride, bromide and formate on the EPR spectra of cytochrome oxidase

Experimental details are as described in the legend to Fig. 9. The % '$g' = 12$' signal present was calculated as described by Brudvig et al. [2], where 100% represents an extrapolation of data from a Yu et al.-type oxidase preparation [36]. EPR spectra were recorded under non-saturating conditions for haem *a* (temperature, 16 K and microwave power, 20 mW), where the $g = 3$ signal could be quantified to approx. 1 haem/enzyme compared to a Cu-EDTA standard using the method of Aasa and Vänngård [43]. Each result is the average from two oxidase samples. The % $g = 6$ signal was calculated using the method of Aasa et al. [44] except that, due to interference from the $g = 4.3$ rhombic iron signal, the double integral was calculated from $g = 6.73$ to $g = 4.86$ rather than $g = 4.67$. As shown by Aasa et al. this value was taken to be 50% of the total double integral and then compared to a metmyoglobin standard (see Ref. 31) run under the same EPR conditions.

Addition	haem *a* *g*-values			% '$g' = 12$'	% $g = 6$
None	3.02	2.22	1.44	30	0.8
Chloride (0.5 M)	2.98	2.24	1.48	30	4
Bromide (0.5 M)	2.98	2.24	1.48	45	2.5
Formate (50 μM)	3.02	2.22	1.44	60	1
Formate (5 mM)	3.00	2.22	1.45	80	1.3

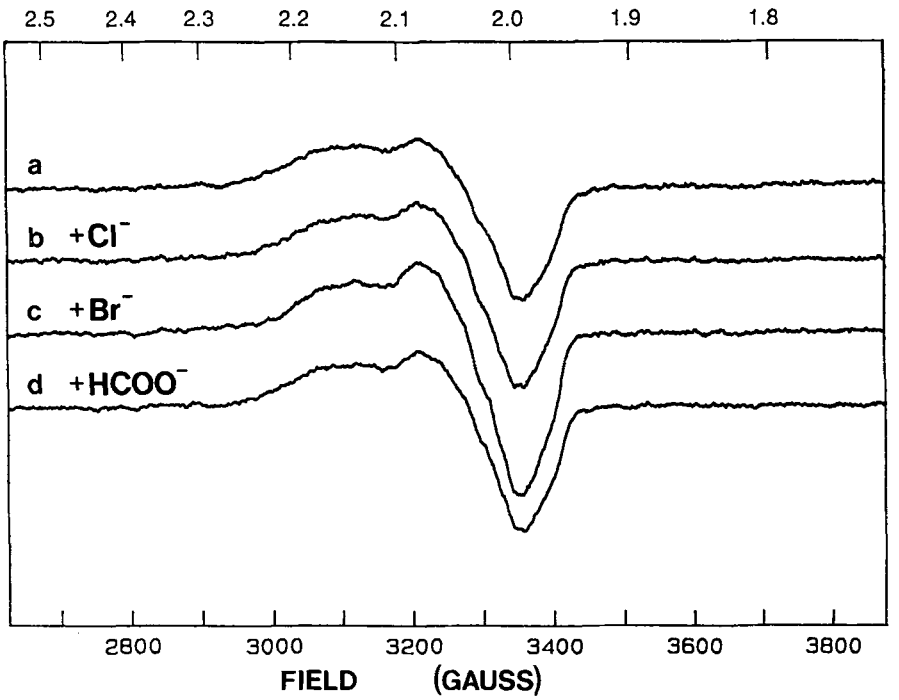

Fig. 10. The effect of chloride, bromide and formate on the copper EPR signals of 'fast' cytochrome oxidase. Samples (a)–(d) correspond to samples (b), (c), (d) and (f) in Fig. 9, respectively. The EPR conditions were as in Fig. 9 except the following: temperature, 70 K; gain, 1E+5; and sweep rate, 7.5 gauss/s.

chloride-induced shifts have been observed for both purified enzyme and submitochondrial particles [31]. It should be noted that the chloride-induced effect is complete within 1 min of its addition. We have not observed the splitting effect of formate on the $g = 3$ signal that Keyhani and Keyhani [18] reported although the concentration used here was lower (5 mM rather than 20 mM).

As well as these shifts in the position of the EPR signal at $g = 3$, there appears to be a considerable change in lineshape under different conditions. The apparent change in lineshape correlates with the presence of the '$g' = 12$' signal and appears to denote the presence of a separate broad signal underlying that at $g = 3$ when a '$g' = 12$' signal is present. Indeed, in the presence of a strong '$g' = 12$' signal there is an increase in the area under the $g = 3$ peak, which complicates the calculation of the magnitude of the '$g' = 12$' signal by reference to the $g = 3$ peak using the method of Brudvig et al. [2]. This is most noticeable when the pH 8.0 control spectrum (a) is compared with the high formate spectrum (f, see also Fig. 7 in Ref. 17). At lower temperatures the effect on the $g = 3$ lineshape is more extreme (not shown), again suggesting that the underlying signal is related to the '$g' = 12$' signal, which is strongly temperature-dependent [32]. The underlying signal can be observed on its own by adding dithionite to formate-ligated enzyme, to reduce Cu_A and haem *a*, and then rapidly freezing the sample before significant reduction of the binuclear centre (Fig. 9g). Under these conditions a broad resonance is seen around $g = 2.9$. A similar spectrum has been

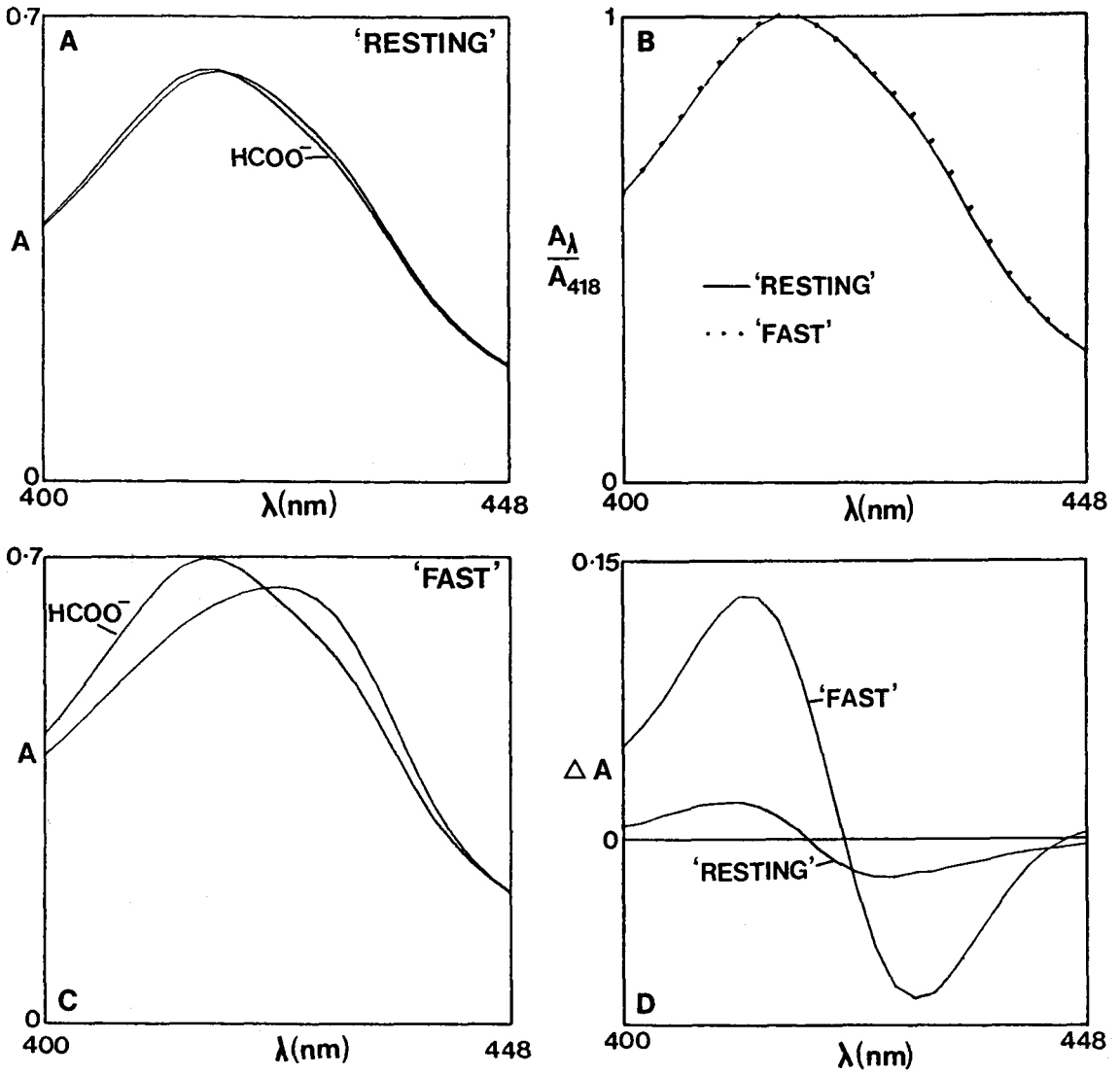

Fig. 11. The effects of formate on the spectra of 'fast' and 'slow' oxidase in the Soret region. Panels A and C shows the spectra of samples of 'slow' and 'fast' (Prep. E) oxidase, respectively, immediately after dilution in 0.2 M potassium Mes buffer (pH 6.5), containing 1 mM potassium EDTA; and after incubation for 5 min with 47 mM potassium formate. Panel B shows the spectra for both samples after incubation with formate normalised with respect to A_{418}. Panel D shows the formate-binding difference spectra.

observed upon addition of ascorbate and cytochrome *c* to formate-ligated enzyme [17] and upon addition of dithionite to 'slow' enzyme [33].

High-field spectra of some of the same samples used to produce the spectra in Fig. 9 were investigated at 70 K to determine whether there was any difference in the Cu EPR signals (Fig. 10). The Cu_A signal was identical in all cases, and no detectable Cu_B signal was observed.

The origin of the '$g' = 12$' signal from 'slow' oxidase is not certain. Hagen [34] has concluded that it represents a $\Delta m_s = 4$ transition within the second non-Kramers doublet of an $S = 2$ system, and has quantified the signal to about one quintuplet per enzyme molecule using $[Fe(H_2O)_6]^{2+}$ as an $S = 2$ standard. It should be noted that the quantitation of the '$g' = 12$' signal in any given oxidase preparation will be dependent on the fraction of the 'slow' form in that preparation. It seems, therefore, that the preparation used by Hagen must have contained a large percentage of the 'slow' form. The data presented here show that the intensity of the '$g' = 12$' signal approaches 100% (using the quantitation devised by Brudvig et al. [2]) in formate-ligated oxidase, i.e., enzyme which binds cyanide slowly and homogeneously.

The effect of formate on 'slow' oxidase

From the data that we have presented on the cyanide-binding kinetics, the dithionite-reduction kinetics and the EPR spectrum of 'fast' oxidase ligated with formate, it can be seen that this form of the enzyme is indistinguishable from the 'slow' form of the enzyme. However, the results presented in Fig. 11 may indicate that the formate-ligated and 'slow' forms of the enzyme show at least one difference. Here we have incubated samples of 'slow' and 'fast' oxidase with formate under identical conditions. The 'slow' oxidase used here, a kind gift from Dr. J.M. Wrigglesworth, showed a negligible fast phase of cyanide binding. Therefore, if the 'slow' and formate-ligated forms of the enzyme had identical properties we would expect no change in the spectrum of the 'slow' oxidase on incubation with formate, but, as shown in panel A, we see a small blue shift. When the difference spectrum for this change is compared to that obtained with 'fast' enzyme, we can see that they are rather different (panel D). There is good agreement between the absorption spectra of both types of oxidase after they have been treated with formate (panel B).

Discussion

The effects of chloride and bromide on 'fast' oxidase

At pH 6.5, the principal effect of chloride on 'fast' oxidase is on the rate of cyanide binding. A form of the enzyme is produced that at pH 6.5 binds cyanide at a rate intermediate between the rate of binding to 'fast' oxidase and the rate of binding to 'slow' oxidase (Fig. 2, panel C), but at pH 8.0 binds cyanide at a rate almost identical to the rate of binding to 'fast' oxidase (Fig. 8 and Table III). The minor changes in the dithionite-reduction kinetics observed during the incubation with chloride (Fig. 6, panels B and C) are consistent with the reversion of a small amount of 'fast' oxidase to the 'slow' form, as is the change in the amplitude of the '$g' = 12$' EPR signal (Fig. 9), although the presence of chloride seems to afford the enzyme some protection against reversion to the 'slow' form. In this case, the amplitude of the difference spectrum induced by chloride in the Soret region is somewhat larger than expected. However, it is qualitatively different to that induced by formate or low pH (Fig. 3), and so we suggest that most of this difference spectrum arises from a spin-state change in a small amount of haem *a*, rather than haem a_3, which is also a possible explanation for the small $g = 6$ EPR signal that is induced by chloride (Fig. 9). If this represents a secondary effect of chloride caused by weak binding then we can also see why it is possible to completely reverse the spectral effect of chloride by removal of the excess by dialysis, even though there is still evidence from the cyanide-binding kinetics that chloride is still bound after dialysis (Fig. 8 and Table III).

At pH 6.5, unlike chloride, the effects of bromide on 'fast' cytochrome oxidase are qualitatively the same as those induced by formate. However, although the quantitative effect of 0.5 M bromide on the cyanide-binding kinetics is almost identical to that produced by 50 μM formate (Fig. 2, panel D), the effect of 0.5 M bromide on the dithionite-reduction kinetics is much less (Fig. 6, panel C). Similarly, the amplitudes of the difference spectrum in the Soret region and the '$g' = 12$' EPR signal induced by 0.5 M bromide are much smaller than those induced by 50 μM formate (Figs. 3 and 9). One way to rationalise this disparity is to assume that bromide is having two effects. The first, like that of chloride, is to lower the rate of cyanide binding, but the second, unlike chloride, is to accelerate the reversion of 'fast' enzyme to the 'slow' form. This interpretation is consistent with the results of the dialysis experiment (Fig. 8 and Table III), where the combined extents of the fast and intermediate phases of cyanide binding observed at pH 8.0 after incubation with bromide (0.085) falls short of the extent of the fast phase in the control incubation (0.111).

It seems, then, that the data presented here are consistent with the binding of chloride or bromide to 'fast' oxidase affecting the rate of cyanide binding but not significantly affecting the spectral properties of haem a_3. Hence, we suggest that chloride and bromide may be acting as ligands for Cu_B.

The heterogeneity in our 'fast' enzyme

Baker et al. (Ref. 6, see also Ref. 35) appear to have obtained complete reversion of their 'fast' oxidase preparations to the 'slow' form after incubation at pH 6.7 and 4 °C for 24 h, but we always see a small residue of fast phase of cyanide binding with our preparations (incubation at pH 6.5 at 22 °C for 24 h). We interpret this, along with other observations, as evidence for the presence of two 'fast' cyanide binding subpopulations in our 'fast' oxidase preparations. We have found that the simplest way to demonstrate this heterogeneity for a given preparation is to measure its formate-binding kinetics, in which case two phases of binding with a 10-fold difference in rate are seen (Fig. 4). For our 'fast' oxidase the extents of these two phases are remarkably constant from preparation to preparation ($\Delta A_{412-432} = 0.125 \pm 0.004$ and 0.099 ± 0.005, relative to A_{422}, for the fast and slow phases, respectively). Further, the formate-binding kinetics are not affected by putting the 'fast' enzyme through a pulsing procedure (reduction using dithionite followed by removal of the excess dithionite by passage through a Sephadex G-25 column equilibrated with buffer at pH 8.5) provided that sufficient time is allowed for the decay of the partially electronated forms of the enzyme formed as the enzyme is exposed to oxygen (about 3 h at 20 °C in the presence of catalase, data not shown).

Baker et al. [6] have already suggested that there is heterogeneity in their 'fast' oxidase preparations because the $g = 6$ EPR signal observed on exposure of the enzyme to NO accounts for only about 20% of it. The work of Brudvig et al. [2] has revealed a similar type of heterogeneity. They identified two subpopulations, other than the 'slow' subpopulation (i.e., the '$g' = 12$' subpopulation), both of which react readily with cyanide, but only one of which exhibits the NO-induced $g = 6$ EPR *. They also noted that a largely 'slow' oxidase preparation produced by the method of Yu et al. [36] (similar to the Kuboyama et al.-type method used here [19]) contained virtually none of the 'NO-induced $g = 6$ subpopulation', but about 24% of the 'EPR-silent' subpopulation. Therefore, we tentatively equate the 'EPR-silent' subpopulation with the minority 'fast' subpopulation reported here, i.e., the subpopulation that resists conversion to the 'slow' form. If this is the case then the term used by Brudvig et al. to describe this subpopulation, i.e., 'oxygenated', may be inappropriate.

* Brudvig et al. used the terms 'resting conformation' and 'oxygenated conformation' for the 'NO-induced $g = 6$ subpopulation' and the 'EPR-silent' subpopulation, respectively. It now appears likely that at least one of these terms is inappropriate, since it is the '$g' = 12$' subpopulation' that has the property of slow intramolecular electron transfer originally associated with 'slow' ('resting') oxidase [10].

Spin state of haem a_3 in 'fast' enzyme

Palmer and co-workers do not subscribe to the view that the blue shift in the Soret absorption band that occurs on conversion of 'fast' to 'slow' oxidase at low pH is due to a spin-state change in haem a_3 [1]. This follows from their conclusion that haem a_3 is high-spin in both 'fast' and 'slow' oxidase, a conclusion which is based partly on the observation that the '655 nm' absorption band is present in 'fast' oxidase and partly on resonance Raman spectroscopy, but mainly on the magnetic susceptibility measurements. It is well established that the low-field and high-field ligands, formate and cyanide, respectively, give rise to oxidase complexes in which haem a_3 is high-spin and low-spin, respectively (see, for example, Ref. 37). Therefore, we find it significant that the binding spectra of cyanide and formate with 'fast' oxidase are essentially the inverse of one another, both in the Soret region and in the alpha region *, since the implication of this observation is that 'fast' enzyme contains an equilibrium mixture of high-spin and low-spin haem a_3 at room temperature. In addition, we feel that evidence already available may be consistent with this view:

(1) The '655 nm' band

The '655 nm' charge transfer band, a marker for high-spin haem a_3, is present in 'fast' oxidase. This could be consistent with haem a_3 being in a mixture of spin states. We would then expect the amplitude of the '655 nm' band to be greater in 'slow' oxidase than in 'fast' oxidase, but at present we do not know if this is so. In any case quantitation of the absorption band is likely to be complicated by the peak shift associated with the conversion of 'fast' oxidase to the 'slow' form [1].

(2) Resonance Raman spectra

The published spectra [35] show that the high-spin marker for haem a_3 (1572 cm^{-1}) [37] is present in 'fast' oxidase, and again this could be consistent with a mixture of spin states. Further, the amplitude of this mode is enhanced relative to the low-spin marker for haem a (1590 cm^{-1}) in both 'slow' and formate-ligated oxidase when they are compared with 'fast' oxidase. Schoonover et al. attribute this enhancement to the blue shift of the Soret absorption band of *all* of the haem a_3 towards the laser wavelength (413.1 nm) in 'slow' enzyme relative to 'fast' enzyme. However, it could equally well be due to a blue shift in *part* of the haem a_3 as the result of a spin-state change.

* With the exception of the region from 630–700 nm, where there is a trough in the cyanide binding spectrum and a differential feature in the formate binding spectrum. The former is caused by the disappearance of the '655' nm band, the latter by a peak shift in this band (Mitchell, R., unpublished results).

(3) Magnetic susceptibility

Baker et al. [6] report that there is no difference in the magnetic susceptibility of 'fast' and 'slow' oxidases when this is measured between 20 and 200 K. This is clear evidence that haem a_3 in 'fast' enzyme is high-spin over this temperature range, but it does not preclude the possibility that there is a thermal mixture of the high-spin and low-spin configurations of haem a_3 at room temperature, in which case the high-spin form would be the ground state [38]. If this is the case then we would predict that the optical spectrum of 'fast' oxidase should be temperature-dependent.

Is the peak shift in the Soret band directly related to the conversion of 'fast' enzyme to the 'slow' form at low pH?

Palmer et al. [1] have reported that when the pH is raised again after the conversion of a 'fast' oxidase preparation to the 'slow' form, the position of the Soret maximum shifts back to the red without any loss in '$g' = 12$' signal and without recovery of the fast cyanide binding form. We do not find this with our preparations of oxidase. For example, we have described an experiment where 'fast' oxidase was incubated at low pH with no additions, or formate, chloride or bromide present, and then dialysed thoroughly at pH 8.0 (Fig. 8 and Table III). For the three cases where we have attributed the blue shift in the Soret band associated with the incubation at pH 6.5 to haem a_3, i.e., no additions, plus formate and plus bromide, we find that the position of the Soret maximum after subsequent dialysis at pH 8.0 does not recover to any great extent (Table III). For chloride, where we attribute the blue shift mostly to haem a, the control position of the Soret maximum (423.8 nm) was recovered after dialysis, consistent with the extent of the fast phase of cyanide binding (compare the control experiment before dialysis with the plus chloride experiment after dialysis.).

Equivalence of the 'resting' and 'slow' forms, and the 'pulsed' and 'fast' forms of oxidase

There seems little question as to the equivalence of the 'resting' and 'slow' forms of cytochrome oxidase. We have found that those preparations which exhibit a slow phase of cyanide binding also show a slow phase of reduction of haem a_3 by dithionite whereas those preparations which exhibit fast monophasic cyanide binding show rapid reduction of haem a_3 that is not kinetically distinct from the reduction of haem a. Further, there is good quantitative agreement between the fraction of enzyme in the 'slow' form as assessed by the slow phase of cyanide binding and the fraction of enzyme in the 'resting' form as assessed by the slow phase of dithionite reduction (see, for example, Fig. 8).

It is less clear whether the 'pulsed' and 'fast' forms of cytochrome oxidase are exactly equivalent. Palmer et al. [6] have suggested that they are not, because 'slow' oxidase that has been pulsed (redox cycled) reverts to the 'slow' form over a period of hours, whereas 'fast' enzyme that has been pulsed does not. They have proposed that this memory of the enzyme for its form before pulsing is due to the involvement of an amino acid modification in the 'fast' to 'slow' conversion [1]. We question whether in fact there is any difference in the tendency of pulsed 'slow' and pulsed 'fast' oxidase to revert to the 'slow' form. It is quite clear from both our own results and those of Palmer et al. that 'fast' oxidase, as prepared, is not permanently in this form because it tends to convert to the 'slow' form if the pH is lowered below about 8. It is also quite clear that the reversion of 'pulsed' enzyme prepared from the 'slow' form shows a similar pH dependence [39]. We therefore suggest that the 'pulsed' and 'fast' enzyme forms are identical.

The effects of formate on 'fast' oxidase and the nature of 'open' oxidase

It is known that the rate of onset of the inhibition of cytochrome oxidase by formate (5–25 mM) is slow at pH 7.4, taking some minutes to complete, but increases in rate as the pH is decreased, and also that any delay in the onset of inhibition can be eliminated by preincubating the enzyme with formate [23]. The effects of formate described here on the oxidised 'fast' enzyme show a similar pH dependence, and, it is noteworthy, that there seems to be no particular disparity between the rate of formate binding to the oxidised form of the enzyme and the delay in the onset of inhibition by formate. Such a disparity does exist for cyanide; the observed rate of binding, even for 'fast' oxidase, is much too slow to account for the rapid onset of inhibition by this ligand. Evidence has been presented that a partially reduced intermediate in the oxidase reaction cycle is responsible for the rapid binding of cyanide [8], and this led to the proposal that there are two conformations of the enzyme, a 'closed' conformation, prevalent in the oxidised enzyme, in which access to the ligand binding site is limited, and an 'open' conformation, which occurs transiently during turnover of the enzyme [9]. The results with formate considerably weaken this hypothesis, since in this case there is no dramatic increase in the rate of formate binding during turnover. Instead, the very rapid binding of cyanide to enzyme which is turning over may arise specifically because of the altered local chemistry of a partially reduced state of the binuclear centre.

The effects of formate on 'fast' oxidase and the nature of 'slow' oxidase

'Slow' oxidase can be considered as a form of the enzyme in which the ligation state of the binuclear centre is different to that in 'fast' enzyme. The pres-

ence of a ligand in 'slow' oxidase decreases the electron affinity of the binuclear centre and also inhibits the binding of cyanide. Reduction of haem a_3/Cu_B displaces this ligand, and on reoxidation it rebinds only slowly, giving rise to the pulsing phenomenon. An alternative proposition is that 'slow' oxidase is a form of the enzyme in which the protein conformation is substantially different from that of 'fast' oxidase such that the intramolecular pathway for electron transfer from haem a/Cu_A to the binuclear centre is disrupted and the binding of cyanide is sterically hindered. The electron is seen as an effector that switches the enzyme from the 'slow' conformer into the 'fast' conformer, i.e., reduction of haem a/Cu_A is required for rapid electron transfer to the binuclear centre.

As far as we are aware, there is no conclusive evidence for or against either model in the literature – the demonstration of structural differences between 'slow' and 'fast' oxidase, either locally, in the binuclear centre [2,40], or more widespread, in the protein conformation [35], is not in itself sufficient to distinguish between the models. Nevertheless, we favour the 'ligation-state' type of model because it offers an elegant description of the behaviour of the enzyme without resort to ad hoc explanations. This has been shown by Bickar et al. [13] who found that the activating effect of cytochrome *c* on the observed rate of electron transfer from haem *a* to haem a_3 can be an intrinsic property of the 'ligation-state' type of model, whereas the 'conformational' model, to explain this effect, requires the additional proviso that cytochrome *c* be an allosteric effector. Furthermore, when they tested a series of modified cytochrome *c* molecules in which the iron was replaced with other metal ions, Bickar et al. found that the acceleration of the intramolecular electron transfer occurred only when the modified cytochrome *c* could undergo reversible redox cycles. This observation is in keeping with the predictions of their model, but not with a 'conformational' model.

From the results presented here it is clear that incubation of 'fast' oxidase with formate produces a form of the enzyme that is indistinguishable from the 'slow' form on the basis of at least three criteria, i.e., cyanide-binding kinetics (Fig. 2), dithionite-reduction kinetics (Fig. 6) and EPR spectrum (Fig. 9). In addition, it has previously been shown that the MCD spectra of formate-ligated and 'slow' oxidases are identical [41]. This similarity constitutes strong evidence in favour of a 'ligation-state' model, since formate is expected to be a low-field haem iron ligand [23].

The only indication of a difference between formate-ligated and 'slow' oxidase is the observation that a slight blue shift in the Soret absorption band is induced in 'slow' oxidase by formate (Fig. 11). The implication of this is that formate displaces the 'slow' ligand from the binuclear centre and thereby shifts the Soret band of high-spin haem a_3 downwards by about 0.5 nm.

The identification of the 'slow' ligand would be a significant step in our understanding of the interconversion of the 'fast' and 'slow' forms of cytochrome oxidase. It is clear that this natural 'slow' ligand copurifies with the enzyme, since the purified enzyme can revert to the 'slow' form without any obvious free ligand being added. Chance and co-workers originally suggested on the basis of EXAFS results that the 'slow' ligand contained sulphur and bridged between Cu_B and haem a_3 [40], but more recently Scott et al. pointed out that the ligand could equally well be chloride, and they presented preliminary results that indicated that this was the case [42]. The data presented here specifically rule out the possibility that chloride (or bromide) is the 'slow' ligand, since it is obvious that the chloride-ligated enzyme does not have the same properties as native 'slow' oxidase. Moreover, the similarity between the formate-ligated oxidase and the 'slow' form strongly suggests that the 'slow' ligand could be a carboxylate group. Two possible sources of a carboxylate-containing ligand are immediately obvious: a glutamate or aspartate residue arising from within the protein or a free fatty acid arising from the lipid carried with the protein during purification.

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