Interconversion of Fast and Slow Forms of Cytochrome *bo* from *Escherichia coli*†

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Received January 10, 1995; Revised Manuscript Received March 16, 1995®

ABSTRACT: The fully oxidized fast form of cytochrome *bo* from *Escherichia coli* is shown to convert spontaneously to a slow form when stored at −20 °C in 50 mM potassium borate, pH 8.5, containing 0.5 mM potassium EDTA. Evidence for the conversion, and that the form produced is analogous to the slow form of bovine heart cytochrome *c* oxidase, comes from (a) decreases in the extents of fast ($k = 1-2 \times 10^3$ M^{-1} s^{-1}) H_2O_2 binding and fast ($k = 20-30$ M^{-1} s^{-1}) cyanide binding; (b) changes in the optical spectrum that are like those induced by formate, i.e., a blue shift in the Soret absorption band, loss of absorbance in the α and β bands, and a red shift in the "630 nm" charge-transfer band; (c) changes in the EPR spectrum that are like those induced by formate, i.e., disappearance of signals at $g = 8.6$ and $g = 3.71$, and appearance of signals at $g \approx 13$, $g = 3.14$, and $g = 2.58$; and (d) appearance of a slow phase of reduction of heme *o* by dithionite. The mutant enyzme E286Q also converts to a slow form under the same conditions, as shown by (a) a decrease in the extent of fast H_2O_2 binding; (b) changes in the optical spectrum like those seen with wild-type enzyme; and (c) changes in the EPR spectrum that are like those induced by formate, i.e., disappearance of signals at $g = 7.3$ and $g = 3.6$ and appearance of signals at $g \approx 13$, $g = 3.18$, and $g = 2.59$. Hence, models for the fast to slow conversion that specifically involve ligation of the high-spin heme or Cu_B by E286 (or by the equivalent residue, E242, in bovine heart cytochrome *c* oxidase) are unlikely to be correct.

Cytochrome *bo* from *Escherichia coli* is a member of the superfamily of terminal oxidases that contain a binuclear heme/Cu center (Saraste, 1990). It catalyzes the four-electron reduction of dioxygen to water and couples this reaction to the generation of a transmembrane proton gradient (Puustinen et al., 1989). Electrons are transferred from ubiquinol, via a low-spin heme (heme *b*), to the site of oxygen binding and reduction, a binuclear metal center consisting of high-spin heme (heme *o*) and a copper atom. To be consistent with the nomenclature developed for bovine heart cytochrome *c* oxidase, which is a member of the same superfamily, this copper atom is referred to as Cu_B, but cytochrome *bo* does not contain an equivalent to the Cu_A center found in cytochrome oxidase.

The problem of heterogeneity in purified preparations of fully oxidized bovine heart cytochrome oxidase (EC 1.9.3.1) is relatively well understood. The properties of the fast form,[1] which is thought to be the form present *in situ*, contrast with those of the slow form, which accumulates when the enzyme is exposed to low pH during the preparation procedure or during storage (Baker et al., 1987; Moody et al., 1991a). The Soret absorption maximum of fast oxidase is at 424 nm, whereas that of slow oxidase is at 417 nm, and slow oxidase shows characteristic EPR signals arising from the binuclear center, at $g \approx 12$ and $g = 2.95$, that are not shown by the fast form (Beinert & Shaw, 1977; Greenaway et al., 1977; Brudvig et al., 1981; Cooper & Salerno, 1992). The binuclear center in the slow form is extremely unreactive, both to inhibitory ligands such as cyanide (Brudvig et al., 1981; Baker et al., 1987) and to electron donors such as hydrogen peroxide (Baker et al., 1987) and carbon monoxide (Morgan et al., 1985). It is also only slowly reduced by dithionite (Wrigglesworth et al., 1988). However, reduction and then reoxidation of the binuclear center (pulsing) is sufficient to return the enzyme to the fast form (Antonini et al., 1977; Brunori et al., 1987).

The properties of the slow form of bovine heart cytochrome oxidase are essentially identical to those of the formate-ligated form of the enzyme (Moody et al., 1991a; Schoonover & Palmer, 1991). Because of this, models have been proposed for the conversion from one form to the other that involve, at least at some stage, a ligand rearrangement in the binuclear center so that either heme a_3 or Cu_B, or both heme a_3 and Cu_B, become ligated by a carboxylate, e.g., a glutamyl or aspartyl residue (Moody et al., 1991a; Schoonover & Palmer, 1991; Cooper et al., 1993; Gullo et al., 1993).

We have previously found that several preparations of cytochrome *bo* purified from *E. coli* showed heterogeneous ligand-binding properties that were consistent with the presence of a slow form of the enzyme (Moody et al., 1993a). However, we were unable to demonstrate the spontaneous conversion of the enzyme to this form. Watmough and colleagues (Cheesman et al., 1993; Watmough et al., 1993) have described a purification method for *E. coli* cytochrome

† This work was funded by the U.K. Biotechnology and Biological Sciences Research Council (Grant GR/J28148). C.E.C. is grateful to the U.K. Medical Research Council for a Training Fellowship.

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® Abstract published in *Advance ACS Abstracts,* May 1, 1995.

[1] We treat this as a single form. However, there is evidence suggesting that there are two populations of enzyme with different properties that are not in rapid equilibrium in fast oxidase (Moody et al., 1991a). However, this point is not yet resolved, and others (Baker & Gullo, 1994) would not agree with this interpretation.

0006-2960/95/0434-6838$09.00/0

bo that produces enzyme that behaves essentially homogeneously with respect to ligand binding. They termed this the fast form of the enzyme, but again were unable to demonstrate a conversion to a slow form. However, they have shown, along with others (Calhoun et al., 1992, 1994; Tsubaki et al., 1993), that formate induces EPR signals from cytochrome *bo* that are like those shown by both the slow and the formate-ligated form of bovine heart cytochrome *c* oxidase.

In the present paper we demonstrate for the first time the spontaneous conversion of *E. coli* cytochrome *bo* from the fast to a slow form analogous to that of bovine heart cytochrome oxidase, and this has allowed us to begin testing the notion that a carboxylate residue is responsible for this conversion. We find that the site-directed mutant enzyme in which a highly conserved glutamate, E286, in putative transmembrane helix VI, is changed to glutamine can still convert to the slow form. Hence, a direct involvement of this residue is eliminated.

MATERIALS AND METHODS

Bacterial Growth. Two strains of *E. coli* were used. RG145 was the source of wild-type cytochrome *bo*, while the mutant enzyme E286Q was expressed in a similar strain, RG129. Both strains lack cytochrome *bd* and overproduce cytochrome *bo* by about 5-fold (Au & Gennis, 1987). Growth conditions were as described before (Moody & Rich, 1994). The content of cytochrome *bo* in the cells could be determined by room-temperature laser-flash photolysis of the CO adduct with the fully reduced enzyme (Brown et al., 1994). For the wild-type enzyme the maximal yield in the cells was at stationary phase, i.e., after 16 h of growth. However, the yield of the mutant enzyme E286Q reached a maximum (about 30% of the yield of wild-type enzyme at stationary phase) during logarithmic growth and then declined. Hence, in this case the cells were harvested after 2−3 h.

Isolation of Membranes and Purification of Cytochrome bo. Bacterial membranes were isolated and washed with urea and then cholate essentially by the method of Matsushita et al. (1986), and the cytochrome *bo* was extracted and purified by the method of Cheesman et al. (1993), all as described before (Moody & Rich, 1994). A value of 225 mM^{-1} cm^{-1} for ϵ_{410nm} of the oxidized form was used for the concentration estimates given in the figure captions. Different preparations of cytochrome *bo* as prepared are referred to using capital letters, e.g., Prep A. Purified enzyme was stored at −196 °C unless otherwise stated.

Pulsing Procedure. A pulsing procedure (Antonini et al., 1997) was used to check that we had a fully oxidized and fully active preparation of cytochrome *bo* [see Moody et al. (1993a)]. The purified enzyme was first reduced with 4 mM sodium ascorbate and 50 μM PMS.[2] Subsequently, the enzyme was incubated at 20 °C for 1 h and finally dialyzed against 50 mM potassium borate, pH 8.5, containing 0.5 mM potassium EDTA and 0.05% Triton X-100 (4 × 100-fold excess for 1 h each at 4 °C) during which time it reoxidized. Different batches of pulsed enzyme from the same preparation are referred to by number, e.g., Prep A1. Pulsed enzyme was stored at −196 °C unless otherwise stated.

[2] Abbreviations: PMS, oxidized 5-methylphenazinium methosulfate; MCD, magnetic circular dichroism.

Reagents. All reagents were purchased from either Sigma (Poole, U.K.) or BDH (Poole, U.K.), except the bacterial growth media, which came from Unipath Ltd. (Basingstoke, U.K.), and Triton X-100 (specially purified for membrane research), which came from Boehringer (Mannheim, Germany). *decyl*-Ubiquinol was prepared by reduction of a solution of the quinone in diethyl ether with aqueous buffered dithionite (Rich, 1981); a 50 mM stock solution in dimethyl sulfoxide containing 10 mM HCl was used.

Measurement of Enzyme Activity. The oxidation of *decyl*-Ubiquinol by cytochrome *bo* at 22−23 °C was monitored using $\Delta A_{280-310nm}$. Stock enzyme was diluted to 2−3 nM in 50 mM potassium phosphate, pH 7.0, containing 0.5 mM potassium EDTA. The reaction was started by adding the quinol. Note that no detergent was present other than the Triton X-100 that came from the diluted stock enzyme (final concentration, $10^{-5}-10^{-4}$%). Because of the lower levels of the mutant enzyme, E286Q, in the original membranes (see above), the detergent:enzyme ratio in samples of the purified mutant enzyme was much higher than in samples of wild-type enzyme. The apparent K_m for the quinol, which is dependent on the [detergent], is less than 5 μM in both cases, and so it was possible, within the solubility limit of *decyl*-ubiquinol (≈50 μM), to determine v_{max}, and hence turnover number, with reasonable accuracy.

Optical and EPR Spectroscopy. Spectroscopy was carried out essentially as described in Moody et al. (1991).

RESULTS

Preparation of Homogeneous Fast[3] Cytochrome bo by a Pulsing Procedure. Our previous study on the ligand-binding properties of several preparations of cytochrome *bo* (Moody et al., 1993a) showed that there is potential for heterogeneity in the enzyme as prepared. We have already described a pulsing protocol for cytochrome *bo*, i.e., a method for reducing the enzyme and then returning it to the fully oxidized state, and have described the reaction of H_2O_2 with the product (Moody & Rich, 1994). With fast enzyme prepared by the method of Cheesman et al. [1993; see also Watmough et al. (1993)] heterogeneity is less of a problem. Nevertheless, our pulsing procedure does lead to a slight increase in the extinction coefficients of the H_2O_2-induced spectral changes. Since we wished to clearly define the properties of the fast form of cytochrome *bo*, we routinely pulsed our purified enzyme preparations and then stored them at −196 °C before use (see Materials and Methods). The pulsing protocol also formed a convenient way of changing the buffer in which the enzyme was stored.

In the pulsing protocol the enzyme is first reduced with ascorbate/PMS and then dialyzed under aerobic conditions. The ascorbate is exhausted during the dialysis, leaving the enzyme containing a mixture of reaction intermediates. The rate at which the enzyme returns to the fully oxidized state is greatly enhanced by the presence of the PMS. The effectiveness of PMS in returning cytochrome *bo* to the fully

[3] We have used the terms fast and slow when applied to bovine heart cytochrome *c* oxidase to describe forms of the fully oxidized enzyme that have distinctive properties [see the introduction and, for example, Palmer (1993) for a good glossary of terms]. Here we have extended this terminology to cytochrome *bo* from *E. coli*. We define a pulsing procedure as one in which the enzyme is either turned over or fully reduced, and then fully oxidized. We take pulsed enzyme, which by definition is fully oxidized, to be in the fast form.

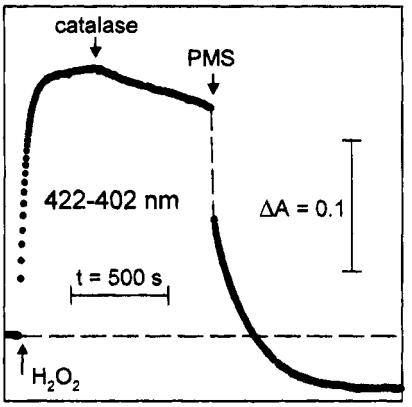

FIGURE 1: Effect of PMS on the decay of the H_2O_2-induced intermediate of cytochrome *bo*. Pulsed enzyme (Prep A1, 71 μM; see Materials and Methods) was diluted to 2.8 μM in 50 mM potassium phosphate, pH 7.0, containing 0.5 mM potassium EDTA, 70 units/mL superoxide dismutase, and 0.05% lauroyl sarcosine. H_2O_2 (20 μM) was added as indicated, and the binding was monitored at 20 °C using $\Delta A_{422-402nm}$. Catalase (80 units/mL) and PMS (50 μM) were added as indicated.

oxidized state is illustrated in Figure 1, which shows the effect of PMS on the decay of an intermediate in the turnover of the enzyme which we have termed F• (Moody & Rich, 1994), in this case quantitatively formed by the reaction of H_2O_2 with the enzyme (Moody et al., 1993b; Watmough et al., 1994; Moody & Rich, 1994). On addition of catalase, to remove excess H_2O_2, F• decays with a rate constant of 2.6×10^{-4} s^{-1}, but when 50 μM PMS is added, after an initial rapid decrease caused by spectral interference from the PMS addition, F• decays to the fully oxidized enzyme with a rate constant of about 5×10^{-3} s^{-1}.

We have previously used PMS to photoreduce mammalian cytochrome *c* oxidase via cytochrome *c* (Moody et al., 1991b). However, even without light, the addition of PMS leads to the reduction of cytochrome *c*, albeit slowly (A. J. Moody, unpublished observation). Hence, while the mechanism by which PMS enhances the decay of the cytochrome *bo* reaction intermediates has not been established, we feel that this is likely to occur by a combination of two effects: (a) catalysis by PMS of the transfer of electrons leaked from the medium to the enzyme, thus completing the turnover of the enzyme and returning it to the fully oxidized state; and (b) catalysis by PMS of the transfer of leaked electrons to oxygen (by disproportionation of the semiquinoid form of PMS and autoxidation of the fully reduced form), thus maintaining the enzyme in the fully oxidized state.

Effects of Storage of Wild-Type Cytochrome bo at −20 °C. (a) Loss of fast H_2O_2 Binding. Figure 2A shows the effect of storage at −20 °C of a batch of fast cytochrome *bo* in 50 mM potassium borate, pH 8.5, containing 0.5 mM potassium EDTA and 0.1% Triton X-100[4] on the kinetics of H_2O_2 binding by the enzyme (Watmough et al., 1994; Moody & Rich, 1994). In all cases the time courses of $\Delta A_{422-402nm}$ can be fitted with a single exponential ($k = 1-2$ M^{-1} s^{-1}) with a superimposed linear drift. As noted before (Moody & Rich, 1994), while the origin of this drift is not

[4] This is a nominal concentration. The enzyme was exchanged into the borate buffer by dialysis. Since Triton X-100 cannot effectively be dialyzed, the [Triton X-100] remains essentially the same as in the original stock enzyme, i.e., 1−2%.

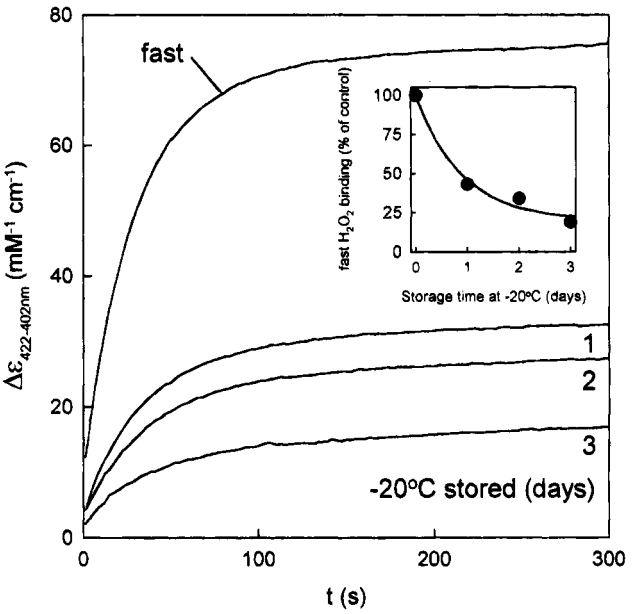

FIGURE 2: Effect of storage at −20 °C on H_2O_2 binding kinetics of cytochrome *bo*. Panel A: A batch of pulsed enzyme (Prep B1, 92 μM) was stored at −20 °C. At the times indicated, the batch was thawed and a sample was taken for measurement of the extent of fast H_2O_2 binding. In each case a baseline spectrum between 390 and 700 nm of buffer (50 mM potassium phosphate, pH 7.0, containing 0.5 mM potassium EDTA) was taken. The sample of cytochrome *bo* was then diluted to 1.8 μM in the buffer and another spectrum was taken. H_2O_2 (20 μM) was added, and binding was monitored at 20 °C using $\Delta A_{422-402nm}$. The time courses were fitted with a combination of a single-exponential absorbance increase and a linear absorbance increase (see text). The extent of fast H_2O_2 binding for a given sample was obtained from the extent of the exponential, and was normalized using $\Delta A_{410-700nm}$ obtained from the spectrum of that sample. Panel B: Plot of extent of fast H_2O_2 binding (% relative to control enzyme) versus storage time at −20 °C derived from the time courses in panel A.

clear, it is unlikely to be caused by H_2O_2 binding. The extent of the H_2O_2 binding phase decreases with storage time (Figure 2B), but no new phase with slower binding kinetics could be detected. In this case the enzyme was stored at −20 °C for 1 day between successive assays. When the same enzyme was stored at −20 °C for only 15 min (i.e., just sufficient to freeze the sample) between successive assays the decrease in the extent of fast H_2O_2 binding was only 16% after 45 min total storage time. It is clear, then, that storage at −20 °C can lead to a large decrease in fast H_2O_2 binding, while the process of freezing at −20 °C and thawing has much less effect. The effect of storage at −20 °C is dependent on preparation; some batches of pulsed enzyme show little or no decrease in fast H_2O_2 binding. However, in all cases loss of fast H_2O_2 binding is observed if a "sink" for electrons is added, e.g., 1 μM PMS and 100 μM potassium ferricyanide (data not shown).

(b) Loss of Fast Cyanide Binding. Figure 3A shows a comparison between the kinetics of cyanide binding to fast cytochrome *bo* and enzyme from the same batch that had been stored at −20 °C. Cyanide induces a red shift in the Soret band of cytochrome *bo*, and this was monitored using $\Delta A_{422-402nm}$. The control enzyme shows almost monophasic cyanide binding ($k_{obs} = 0.10$ s^{-1} with 4 mM KCN; equivalent to $k = 25$ M^{-1} s^{-1}) for which $\Delta\epsilon_{422-402nm} = 106$ mM^{-1} cm^{-1}. The −20 °C stored enzyme shows a binding phase with similar kinetics, but the extent is only 20% of the control.

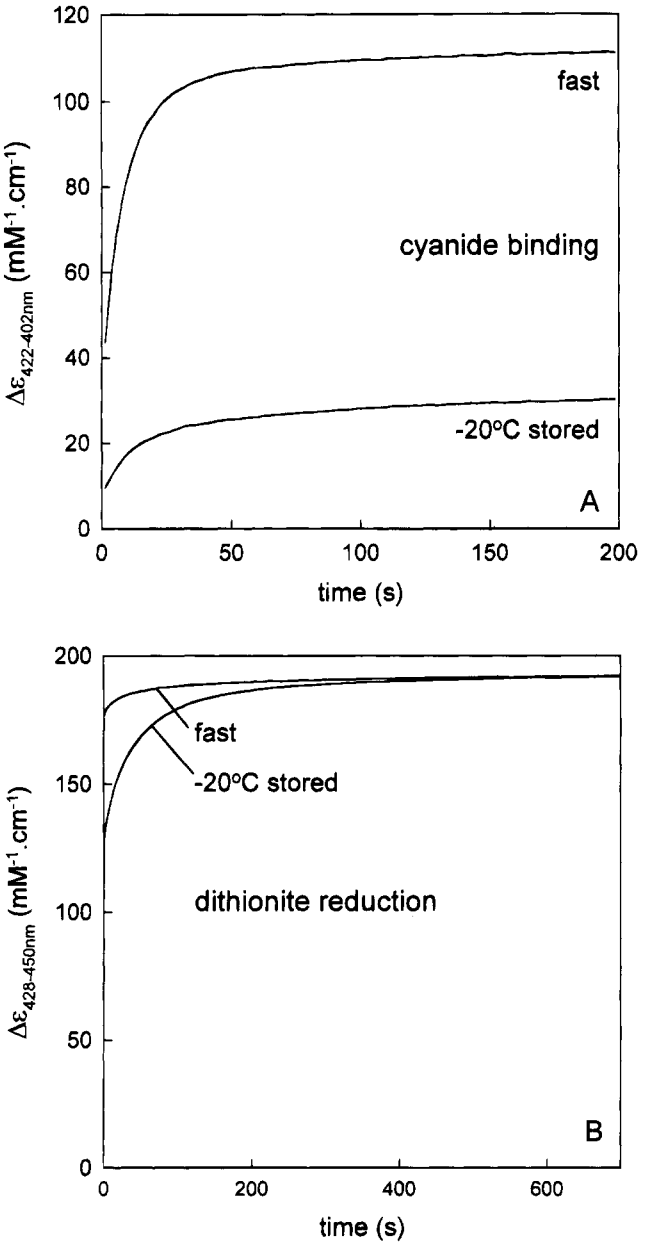

FIGURE 3: Effect of storage at −20 °C on the cyanide binding kinetics and the dithionite reduction kinetics of cytochrome *bo*. In each case a baseline spectrum between 390 and 700 nm of buffer (50 mM potassium phosphate, pH 7.0, containing 0.5 mM potassium EDTA) was taken; the sample of cytochrome *bo* was then diluted to 1−2 μM in the buffer at 20 °C, another spectrum was taken, and either KCN (4 mM) or sodium dithionite and PMS (10 mM and 1 μM, respectively) were added. The time course of cyanide binding was monitored using $\Delta A_{422-402nm}$; that of dithionite reduction was monitored using $\Delta A_{428-450nm}$. The time courses were normalized using $\Delta A_{410-700nm}$ obtained from the spectrum of the enzyme before the addition of cyanide or dithionite/PMS. Two types of enzyme were tested: fast enzyme (Prep B2, 73 μM) and enzyme from the same batch that had been stored at −20 °C for 15 days in the presence of 1 μM PMS and 100 μM potassium ferricyanide and for which fast H_2O_2 binding had decreased to 13% of the control. Panel A: Cyanide binding kinetics. Panel B: Dithionite reduction kinetics.

The appearance of a new phase, with slower binding kinetics, could not be detected at the [KCN] used.

(c) Changes in Dithionite Reduction Kinetics. Figure 3B shows a comparison between the kinetics of reduction by 10 mM dithionite (with PMS as a mediator) of fast cytochrome *bo* and −20 °C stored enzyme. The appearance

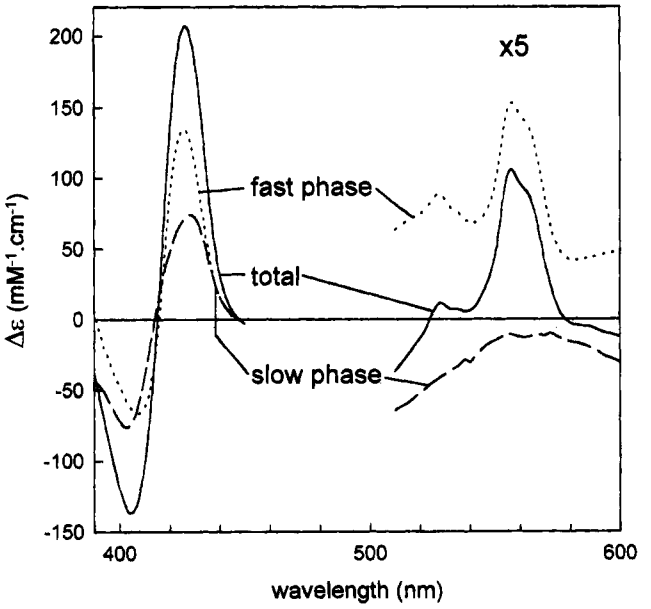

FIGURE 4: Dithionite-reduced minus air-oxidized difference spectra. Pulsed cytochrome *bo* (Prep B2, 73 μM) that had been stored at −20 °C for 15 days in the presence of 1 μM PMS and 100 μM potassium ferricyanide was used. Fast H_2O_2 binding by this enzyme had decreased to 13% of the control. It was diluted to 1.4 μM in 50 mM potassium phosphate, pH 7.0, containing 0.5 mM potassium EDTA at 20 °C, and a baseline spectrum was taken. Sodium dithionite and PMS (10 mM and 1 μM, respectively) were then added. The spectrum shown with a continuous line was taken about 5 min after the dithionite/PMS addition. The dotted and dashed spectra were deduced using a time course of $\Delta A_{424-402nm}$ and represent the kinetic spectra of the fast (within mixing time) and slow reductive phases, respectively (see Figure 3B and text). These spectra are displaced on the *y*-axis for clarity.

of the Soret band at 428 nm from the ferrous hemes was monitored using $\Delta A_{428-450nm}$. With the control enzyme, over 90% of final extent of this signal is developed within the dead time for the mixing of the dithionite with the enzyme (<5 s), but 10 min is required for full reduction. With the −20 °C stored enzyme 10 min is again required for full reduction, but less than 70% of the signal is developed within the dead time. The remaining signal appears in a biphasic manner (65% at 0.033 s^{-1}; 35% at 0.007 s^{-1}), and the spectral changes associated with this slow reductive process are consistent with the reduction of only the high-spin heme, heme *o*; i.e., they are characterized by a trough at 403 nm and a peak at 428 nm in the Soret region, and a broad peak of low amplitude centered about 564 nm in the visible region (Puustinen et al., 1992). This is illustrated in Figure 4.

(d) Spectral Changes. Figure 5 shows a comparison between the near-UV/visible absorption spectra of fast cytochrome *bo* and enzyme from the same batch that had been stored at −20 °C for 1 day. The Soret band of the −20 °C stored enzyme is blue-shifted to 406.5 nm relative to the control at 407.5 nm. In addition, there is a loss of absorbance in the region of the α and β bands (520−580 nm), and the charge-transfer band associated with ferric heme *o* is shifted to the red by about 4 nm (from 628 nm in the control).

Figure 6 shows X-band EPR spectra. The spectrum of fast enzyme has characteristic signals at $g \approx 8.6$ and $g = 3.71$ which are similar to those reported before in enzyme as prepared (≈9.1 and 3.74, respectively; Watmough et al.,

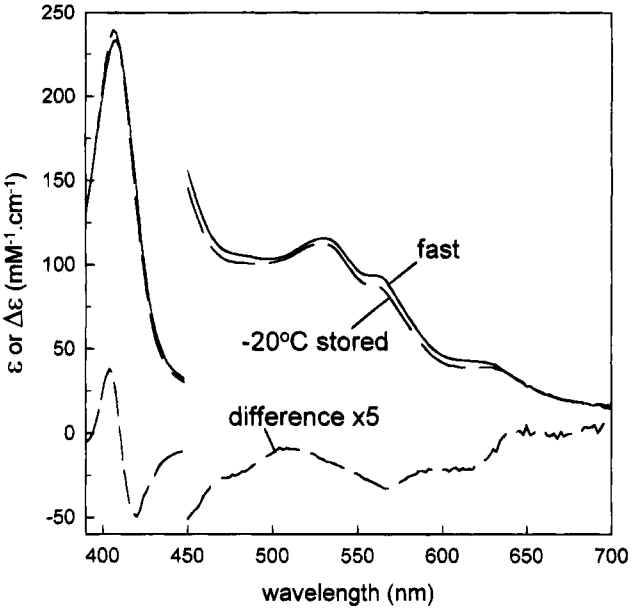

FIGURE 5: Near-UV/visible absorption spectra of fast cytochrome *bo* and enzyme from the same batch that had been stored for 1 day at −20 °C. Enzyme (Prep B1, 92 μM) was diluted to 1.8 μM in 50 mM potassium phosphate, pH 7.0, containing 0.5 mM potassium EDTA. The upper dashed spectrum is of the −20 °C stored enzyme. The lower dashed spectrum is the difference between this and the spectrum of the control enzyme.

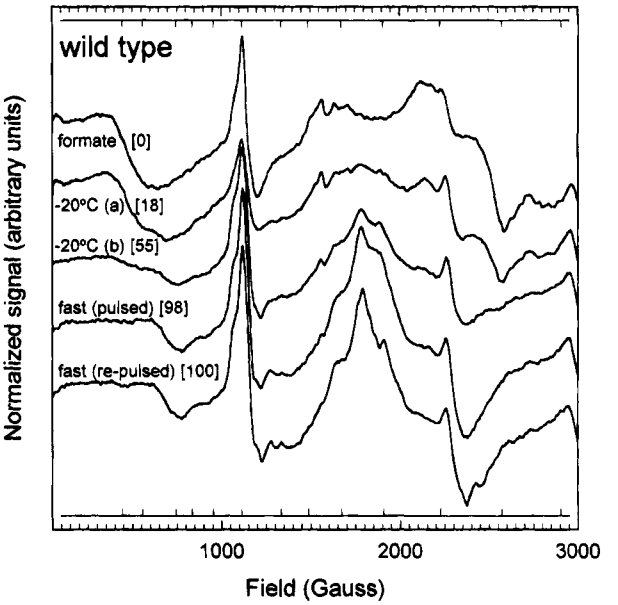

FIGURE 6: X-band EPR spectra of a series of wild type cytochrome *bo* samples. The EPR conditions are as follows: temperature, 5.5 K; microwave power, 200 mW; modulation amplitude, 10 G; modulation frequency, 100 kHz; microwave frequency, 9.35 GHz. Spectra were taken sequentially on the same day, and matched EPR tubes were used. The enzyme samples, as indicated, are as follows: formate, Prep B3, incubated with 90 mM potassium formate for 4 h at 20 °C; −20 °C (a), Prep C1, stored at −20 °C for 14 days; −20 °C (b), Prep. B3, stored at −20 °C for 7 days; fast (re-pulsed), Prep. B3, stored at −20 °C for 7 days and then re-pulsed; and fast (pulsed), Prep B3. The values in square brackets are the fraction of fast H_2O_2 binding for each sample. The [enzyme] was in the range 62−74 μM and was determined by taking the absorption spectrum of each sample before the samples were frozen in EPR tubes. The absorbances at 410 and 700 nm are essentially isosbestic for the spectral changes induced by storage at −20 °C. Hence, the factor $1/\Delta A_{410-700nm}$ was used to normalize the EPR spectra.

1993). These signals have been attributed to an $S = 2$ system arising from a magnetic interaction between heme *o* and Cu_B. In enzyme from the same batch that had been treated with formate they are replaced by characteristic signals at $g \approx 13$,[5] $g = 3.14$, and $g = 2.58$. These have also been reported before [≈13, 3.20, and 2.60, respectively, Watmough et al. (1993); see also Calhoun et al. (1992, 1994)] and again are attributed to the binuclear center acting as an integer-spin system. In addition, weak low-spin heme signals can be seen at $g \approx 3.0$ and 2.2. Under more appropriate EPR conditions (10 K and 2 mW microwave power; data not shown) the low-spin signals are seen clearly at $g = 2.98$, 2.24, and 1.50 (shifted to 2.95, 224, and 1.46 in the presence of 90 mM potassium formate). The signal at $g \approx 6$ is caused by a small percentage of high-spin heme, and may arise from a population of damaged enzyme in which the coupling between heme *o* and Cu_B is broken (see e.g. Moody et al., 1991a).

The spectra of samples of cytochrome *bo* that have been stored at −20 °C are essentially hybrids of the spectra of the fast and formate-treated enzymes, and on this basis a deconvolution matrix based on two "triple-field" measurements can be devised (Table 1). The signal at 650 G minus the weighted average of the signals at 350 and 1000 G is characteristic of the formate-type spectrum, while the signal at 1800 G minus the weighted average of the signals at 1450 and 2150 G is characteristic of the fast-type spectrum. Figure 8 shows a plot of the fraction of formate-type spectrum obtained this way versus the fraction of fast H_2O_2 binding. This plot shows a good linear correlation.

Table 1: A Deconvolution Matrix (Rich et al., 1987) of Relative Signals from Two Triple-Field Measurements for the Fast-Type and Formate-Type EPR Spectra of Wild-Type Cytochrome *bo* and the Mutant, E286Q (Shown in Square Brackets)

	650 − (350, 1000) G[a]	1800 − (1450, 2150) G[b]
fast-type spectrum	−0.040 [−0.015]	1[c]
formate-type spectrum	1	0.067 [0.065]

[a] The signal at 650 G minus the weighted average of the signals at 350 and 1000 G, i.e., $\Delta S_T = S_C - (S_L + ((S_H - S_L)((F_C - F_L)/(F_H - F_L))))$, where F_C, F_L, and F_H are the field values 650, 350, and 1000 G, respectively, and where S_C, S_L, and S_H are the respective signals at these field values. This is an adaptation of the triple-wavelength method that we have used before with absorption spectra (Moody & Rich, 1990). [b] The signal at 1800 G minus the weighted average of the signals at 1450 and 2150 G. [c] The use here of matrix deconvolution is based on the assumption that the observed EPR spectra are simple linear combinations of two base spectra, the fast type and the formate type. The method involves the solution, by matrix inversion, of two simultaneous equations of the form (1) observed signal at *y* G = Σ(signals from each component at *x* G) × (signal size at *y* G for each component relative to that at *x* G) and (2) observed signal at *x* G = Σ(signals from each component at *x* G).

After pulsing, the altered EPR spectrum of −20 °C stored enzyme reverts to that characteristic of fast enzyme (Figure 6, re-pulsed).

Some Properties of the Mutant Cytochrome bo, E286Q. We find that the mutation in which glutamate-286 is changed

[5] The *g*-values given are from spectra taken at 9 GHz. However, the equivalent values in bovine heart cytochrome *c* oxidase are dependent on microwave frequency used (Dunham et al., 1983; Hagen, 1982).

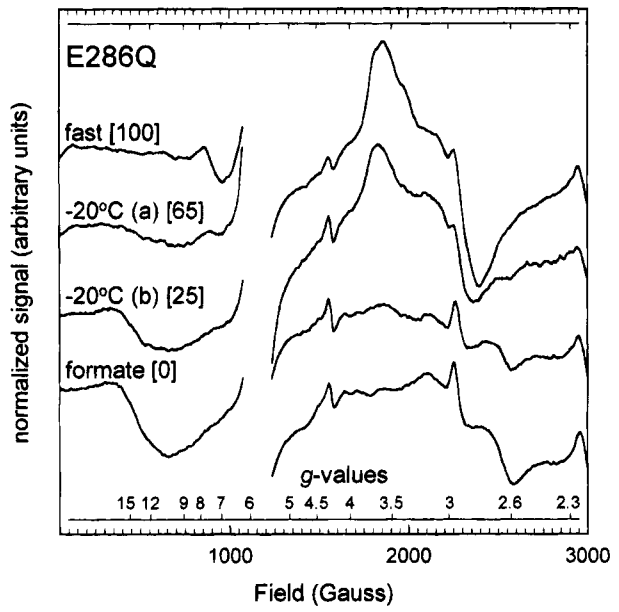

FIGURE 7: X-band EPR spectra of a series of mutant (E286Q) cytochrome *bo* samples. The EPR conditions were as in Figure 6. The enzyme samples, as indicated, are as follows: fast, Prep D; 20 °C (a), Prep D1, stored at −20 °C for 1 day; −20 °C (b), Prep. D1, stored at −20 °C for 7 days in the presence of 1 μM PMS and 100 μM potassium ferricyanide; formate, Prep D, incubated with 90 mM potassium formate for 4 h at 20 °C. The values in square brackets are the fraction of fast H_2O_2 binding for each sample. The [enzyme] was in the range 46−61 μM. As in Figure 6, the factor $1/\Delta A_{410-700nm}$ was used to normalize the spectra.

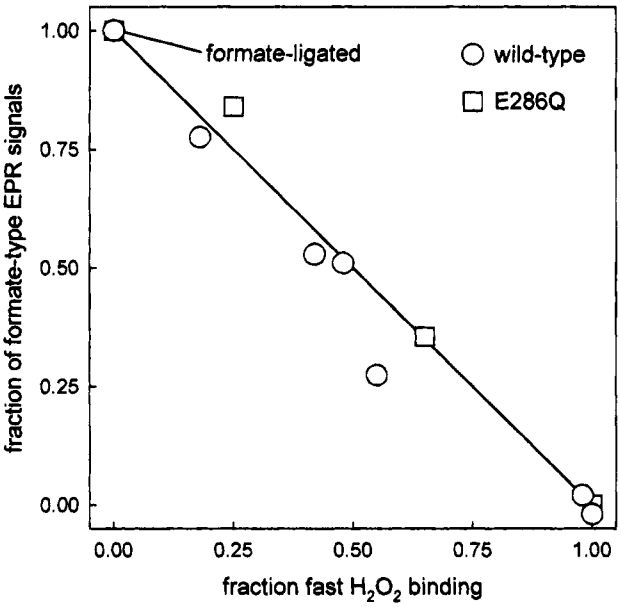

FIGURE 8: Correlation between fast H_2O_2 binding and the type of EPR spectrum. The fraction of formate-type (○ or □) spectrum was determined by matrix deconvolution (Rich et al., 1987) of the normalized EPR spectra (see, e.g., Figures 6 and 7) on the basis of two triple-field measurements. The deconvolution matrices for wild-type enzyme (○) and for the mutant enzyme, E286Q (□), are shown in Table 1. See text for further details. The fraction of fast H_2O_2 binding was obtained as described in the Figure 2 caption. The straight line is a perfect negative correlation.

to glutamine (E286Q) has little or no effect on the catalytic activity of the purified enzyme; maximal turnover numbers for wild type and E286Q are 592 ± 29 e^- s^{-1} (SEM, $n = 5$) and 631 ± 50 e^- s^{-1} (SEM, $n = 7$), respectively, using *decyl*-ubiquinol. We find a large difference in the apparent K_m for the quinol between E286Q (3.5 ± 1.1 μM, SEM, $n = 7$) and the wild-type enzyme (much less than 2.5 μM), but this is probably an artifact caused by a difference in detergent: enzyme ratio (see Materials and Methods). The catalytic activity in the presence of 0.05% dodecyl β-D-maltoside was about 2-fold greater for both enzymes, but because the apparent K_m in this case was close to the solubility limit of the *decyl*-ubiquinol (about 100 μM), v_{max} could not be determined as accurately.

The Soret maximum of pulsed E286Q is at 408 nm, whereas that of the pulsed wild-type enzyme is between 407 and 407.5 nm. It binds H_2O_2 with similar kinetics, and the H_2O_2-induced spectral changes are essentially the same, but $\Delta\epsilon_{422-402nm}$ is found to be 46 mM^{-1} cm^{-1}, whereas this value reaches 86 mM^{-1} cm^{-1} for pulsed wild-type enzyme (Moody & Rich, 1994).

Effect of Storage at −20 °C on the EPR Spectrum of the Mutant Cytochrome bo, E286Q. As prepared, purified E286Q showed the same extent of fast H_2O_2 binding as after pulsing. Storage of pulsed purified E286Q at −20 °C leads to a loss of fast H_2O_2 binding, especially if an electron sink (PMS/ferricyanide as before) is present. Figure 7 shows the X-band EPR spectra of a set of E286Q samples. The differential feature at $g = 7.3$ in the spectrum of the fast sample distinguishes E286Q from the wild-type enzyme. Together with a signal at $g = 3.6$, this is, presumably, another manifestation of an integer-spin system arising from the binuclear center. The spectrum of formate-treated E286Q is essentially identical to that of formate-treated wild-type enzyme, with signals at $g \approx 13$, $g = 3.18$, and $g = 2.59$. As with wild-type enzyme, under the appropriate conditions (10 K and 2 mW microwave power; data not shown) low-spin heme signals at $g = 2.95$, 2.24, and 1.50 (shifted to 2.96, 2.23, and 1.48 in the presence of 90 mM potassium formate) are observed. A small amount (though 2−3-fold greater than with wild type) of a high-spin heme signal at $g \approx 6$ is also seen.

The spectra of samples of E286Q that have been stored at −20 °C are, as with wild-type enzyme, clearly hybrids of the spectra of the fast (as prepared) and formate-treated enzymes. The fast-type and formate-type spectra can be deconvoluted using a matrix based on the same two triple-field measurements that were used for the wild-type enzyme (Table 1). As with the wild-type enzyme, there is a good linear correlation between the fraction of the formate-type spectrum and the fraction of fast H_2O_2 binding remaining (Figure 8).

DISCUSSION

We have examined the effects of storage at −20 °C in borate buffer on the properties of the cytochrome *bo* from *Escherichia coli*; storage in this manner is a convenient method for converting bovine heart cytochrome oxidase to the slow form (Moody et al., 1994a). The clear conclusion from this work is that storage at −20 °C produces changes in a range of properties of cytochrome *bo* that are analogous to the changes seen when the fast form of cytochrome oxidase is converted to the slow form (see Table 2 for a summary), and that these changes can be reversed by redox-cycling (pulsing) the enzyme. Perhaps the most definitive change is the appearance of EPR signals at $g \approx 13$, $g = 3.14$, and $g = 2.58$ (Figure 6) that are identical to the signals

Table 2: Comparison of the Properties of the Fast and Slow Forms of *E. coli* Cytochrome *bo* with the Analogous Forms of Bovine Heart Cytochrome *c* Oxidase

	E. coli cytochrome *bo*[a]			bovine heart cytochrome *c* oxidase[b]		
	fast	slow	formate-ligated	fast	slow	formate-ligated
Soret maximum	407−407.5 nm	< 406.5 nm	406 nm	424 nm	417 nm	417 nm
Integer-spin EPR signals	$g \approx 9$ and $g = 3.7$	$g \approx 13$, $g =$ 3.1 and 2.6	$g \approx 13$, $g =$ 3.1 and 2.6	none	$g \approx 12$ and $g = 3.0$	$g \approx 12$ and $g = 3.0$
Reduction by dithionite	hemes *b* and *o*, fast and simultaneous	heme *b* fast; heme *o* slow and biphasic, $k = 0.033$ and 0.007 s^{-1} 65% fast	not known	hemes *a* and a_3, fast and simultaneous	heme *a* fast; heme a_3 slow and biphasic, $k = 0.028$ and 0.008 s^{-1} 24% fast[c]	heme *a* fast; heme a_3 slow and biphasic, $k = 0.024$ and 0.008 s^{-1} 24% fast[d]
Cyanide binding	fast, $k = 20-30$ M^{-1} s^{-1} with 4 mM KCN	not detected, presumed $k < 0.3$ M^{-1} s^{-1}	slow, $k < 0.3$ M^{-1} s^{-1} [e]	fast, $k = 1-2$ M^{-1} s^{-1} with 20−40 mM KCN	slow, $k = 0.01-0.02$ M^{-1} s^{-1}	slow, $k =$ 0.01−0.02 M^{-1} s^{-1}
H_2O_2 binding	fast, $k = 1-1.5 \times 10^3$ M^{-1} s^{-1} [f]	not detected, presumed $k < 20$ M^{-1} s^{-1}	not known	fast, $k = 4 \times 10^2$ M^{-1} s^{-1} [g]	slow, k presumed to be < 4 M^{-1} s^{-1} [h]	not known

[a] Data for cytochrome *bo* are taken from the present work unless otherwise indicated. [b] Data for cytochrome oxidase are taken from Moody et al. (1991) unless otherwise indicated. [c] Cooper et al. (1993), 25 mM dithionite at pH 6.5. [d] A. J. Moody, unpublished observation, 20 mM dithionite at pH 6.5. [e] Moody et al. (1993a). [f] Moody et al. (1993b); Watmough et al. (1994); Moody & Rich (1994). [g] Weng & Baker (1991). [h] Baker et al. (1987).

that are seen when the enzyme is incubated with formate (Calhoun et al., 1992, 1994; Watmough et al., 1993; Tsubaki et al., 1993), but the appearance of two slow phases in the dithionite-reduction kinetics (Figure 3B) in which only heme *o* is reduced (Figure 4) is also persuasive. These phases and their rate constants are remarkably similar to data obtained for bovine heart cytochrome oxidase (Cooper et al., 1993; see Table 2). So, too, are the changes in the absorption spectrum (Figure 5) which, as with cytochrome oxidase, are like those seen when the enzyme is treated with formate[6] (Watmough et al., 1993).

Previous attempts to demonstrate the spontaneous conversion of cytochrome *bo* to a slow form have failed (Moody et al., 1993a; Watmough et al., 1993), although there was evidence for the presence of such a form in enzyme as prepared (Moody et al., 1993a). Our experience with purified bovine heart cytochrome *c* oxidase is that the rate and extent of the conversion of the enzyme from the fast to the slow form depend upon the medium in which the enzyme is dissolved (A. J. Moody, unpublished observation). One possible reason for this is that the level of electron "leakage" through the enzyme varies and, hence, the rate of conversion of the slow form back to the fast form varies. This appears also to apply to cytochrome *bo*, and to some extent the problem is alleviated by including an electron sink such as PMS/ferricyanide. However, even with an electron sink, we have not as yet achieved quantitative conversion of the enzyme to the slow form. This is also remarkably like the behavior of bovine heart cytochrome *c* oxidase [see Moody et al. (1991a, 1994a)].

The similarity between the slow form of bovine heart cytochrome oxidase and the form induced by formate treatment led to the suggestion that a carboxylate residue in the enzyme itself might be involved in the conversion to the slow form (Moody et al., 1991a; Schoonover & Palmer, 1991). Of the many potential ligands tested [see, e.g., Schoonover & Palmer (1991)], it appears that only formate can quantitatively induce the $g = 12$ EPR signal, while chloride, which has been implicated in the fast to slow conversion (Scott et al, 1988), clearly does not, and in any case, chloride induces ligand-binding properties that are different from those of the slow enzyme (Moody et al., 1991a, 1994b).

MCD studies on formate-ligated cytochrome *bo* clearly suggest a direct ligation of the formate to heme *o* (Cheesman et al., 1994). Hence, the simplest view of the fast to slow conversion is that a ligand rearrangement in the binuclear center allows a carboxylate residue to directly ligate heme a_3 (Moody et al., 1991a; Moody, 1991). A glutamate residue in this subunit [E242 in *Bos taurus*; E286 in *E. coli*; unless otherwise indicated, sequence information was taken from Saraste (1990)], in putative transmembrane helix VI, is conserved throughout the known heme/Cu terminal oxidase superfamily with the exceptions of enzymes from *Sulfolobus acidocaldarius* (SoxB product; Lübben et al., 1992) and *Thermus thermophilus* (*caa*$_3$; Fee et al., 1993), and this residue was considered a prime candidate for the "slow" ligand (Moody et al., 1993; Brown et al., 1993). We show here, however, that a mutant of cytochrome *bo* in which E286 is changed to glutamine can convert spontaneously to a slow form in the same way as the wild-type enzyme. Hence, a direct involvement of E286 in the fast to slow conversion is effectively ruled out.

Since the mutation E286Q does not affect the electron-transfer activity (present work; Thomas et al., 1993), the proton pumping activity (Thomas et al., 1993), or the fast to slow conversion, it is unclear what the "function" of E286 is. However, our finding that levels of E286Q plateau and then decline during growth (see Materials and Methods) suggests that E286 may have a structural role. In current structural models [e.g., Brown et al. (1993)] it is easier for E286 to face away from the binuclear center. In this position it could be involved in intersubunit interactions, and perhaps in the stabilization of the dimeric form of the enzyme.

The notion of the involvement of a carboxylate residue in the fast to slow conversion is not of course eliminated by the data presented here because there are other candidates,

[6] It is clear, with hindsight, that the enzyme that we used before for formate-binding studies contained significant levels of the oxygen intermediate which we have termed F• (Moody & Rich, 1994) and, hence, that the formate-binding spectrum shown in Moody et al. (1993) is contaminated badly by the decay of this intermediate. In our view, this also applies to the binding spectrum shown by Ingledew et al. (1993).

e.g., a highly conserved aspartate residue (D407 in *E. coli*; D364 in *B. taurus*; D373 in *T. thermophilus* caa_3; N361 in *S. acidocaldarius* SoxB product) in the loop between helices IX and X; there is growing evidence for an interaction between residues in this loop and the binuclear center (Meunier et al., 1993; Hosler et al., 1994). There are also highly conserved carboxylate residues (D256 in *E. coli*; D213 in *B. taurus*; E222 in *T. thermophilus* caa_3; and absent in *S. acidocaldarius* SoxB product) in the loop between helices V and VI (D256 in *E. coli*; D213 in *B. taurus*; E222 in *T. thermophilus* caa_3; and absent in *S. acidocaldarius* SoxB product) and in the loop between helices II and III (D135 in *E. coli*) which, in current structural models, could be placed close to the binuclear center. The latter residue has been implicated in the mechanism of proton translocation (Thomas et al., 1993). In addition to carboxylate residues from the protein itself, the possibility that fatty acids that are copurified with the enzyme are involved (Moody et al., 1991a) has not been eliminated. However, it is clear that bicarbonate can be eliminated as a candidate for the slow ligand (Moody et al., 1994b).

The results presented here show clearly that the fast/slow form interconversion, rather than being a phenomenon peculiar to bovine heart cytochrome *c* oxidase, is also a feature of *E. coli* cytochrome *bo*. There are further examples of heme/Cu terminal oxidases where it is likely that a slow form exists. For example, Fujiwara et al. (1992) found that cytochrome baa_3 isolated from *Pseudomonas aeruginosa* showed a well-defined slow phase of dithionite reduction with the spectral characteristics of heme a_3. Geier et al. (1994) have found that a fraction of cytochrome aa_3 purified from *Saccharomyces cerevisiae* shows slow cyanide binding kinetics. Sone and colleagues (Sone & Nicholls, 1985; Sone et al., 1994) have also reported slow phases of cyanide binding by cytochromes *cao* and caa_3 isolated from the thermophilic bacterium *Bacillus* PS3 but interpret the kinetics in terms of a single population of enzyme that binds cyanide in a two-step reaction. This interpretation was originally applied to the bovine heart enzyme (Nicholls et al., 1972). However, the current consensus, when a slow phase of cyanide binding is shown by a preparation of bovine heart enzyme, is that this is an indication of the presence of a subpopulation of the slow form of the enzyme (Baker et al., 1987). We feel that it is a more likely explanation for the slow phases of cyanide binding seen with the PS3 enzymes, and, indeed, is consistent with the observation that the enzymic activity of cytochrome caa_3 from PS3 is greatly enhanced after the enzyme is redox cycled (Sone et al., 1984).

To our knowledge the presence of a $g = 12$-type EPR signal as a result of spontaneous conversion of the enzyme to a slow form has not, until now, been convincingly demonstrated in a heme/Cu terminal oxidase other than mammalian cytochrome oxidase. Geier et al. (1994) have found some indication of a $g = 12$ signal in enzyme from *S. cerevisiae*, and as noted before by Watmough et al. (1993), broad signals in the $g = 3-4$ region have been seen in the EPR spectrum of cytochrome caa_3 from *T. thermophilus* (Fee et al., 1980, 1993). Similar signals can be seen in the EPR spectrum of cytochrome caa_3 from *Bacillus* PS3 (Sone et al., 1984), and in both cases they have been interpreted as arising from low-spin heme *c*, but another explanation is that at least part arises from integer-spin systems. However, in the absence of the more diagnostic low-field region, it is not possible to assess whether slow forms of these enzymes are involved.

ACKNOWLEDGMENT

For technical assistance we thank Alan Jeal (bacterial growth and harvesting), Robert Harper (membrane preparation), and Sally Madgwick (activity assays). We are also grateful to Professor R. Cammack for the use of the EPR spectrometer at King's College, London, and to Andy White and Dr. Jasvinder Shergill for assistance with a technical problem with the spectrometer.

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BI950047A