INTRODUCTION

The cytochrome (cyt) $b_{6}f$ complex is situated in the thylakoid membrane, where it mediates linear electron transfer between photosystems II and I and performs cyclic electron transfer. Both processes result in proton translocation across the membrane, creating an electrochemical gradient for ATP synthesis. Homologous to the mitochondrial cytochrome $bc_{1}$ complex, cyt $b_{6}f$ incorporates cytochrome $f$ (a c-type cytochrome) and an Fe$_{2}$S$_{2}$ protein, constituting the linear electron pathway from hydroplastoquinone to plastocyanin, and cytochrome $b_{6}$ containing the haem groups $b_{1}$ and $b_{2}$ involved in cyclic electron transfer. Unlike the mitochondrial complex, the $b_{6}f$ complex also contains a chlorophyll (chl) $a$, a $\beta$-carotene (car), and a fourth haem, haem $c_{1}$ (reviewed by Allen (2004)). The presence of haem $c_{1}$ at the stromal quinone binding site came as a surprise when the X-ray crystal structure was solved last year, for cyt $b_{6}f$ purified from the unicellular green alga Chlamydomonas reinhardtii (Stroebel et al 2003) and from the thermophilic cyanobacterium Mastigocladus laminosus (Kurisu et al 2003). The Fe of haem $c_{1}$ is penta-coordinated, with water (or hydroxide) the only axial ligand. This indicates a high-spin configuration of the haem, which would explain why it has not been predicted from previous spectroscopic studies.

RESULTS AND DISCUSSION

1.7-K Absorption and MCD Spectra of cyt $b_{6}f$.

In Fig. 1A, the 1.7-K absorption spectrum of cyt $b_{6}f$ is displayed, and the origin of each spectral contribution specified. Chl $a$ dominates the spectrum, with a strong Soret band at 420 nm and the Q$_{y}$ transition at 670 nm. The vibrational side-bands of the Q$_{y}$ peak and the Q$_{x}$ transition are present in the 550–650 nm region. These features identify the pigment as a regular chl $a$. The $\beta$-car absorption is also visible, at 488 nm.

The peaks at 548 and 551.5 nm have been assigned to the Q$_{y}$ transitions of the reduced form of cyt $f$ (Schoepp et al 2000). The vibrational side-bands are discernible in the 500–550 nm region. The presence of these features establishes that in our preparation, cyt $f$ is reduced to a significant extent, while the other haem groups are oxidized. The absorption features of oxidized, low-spin haem are spread over 400–600 nm, but are too weak to stand out under the chl $a$ Soret band. However, these features are accompanied by strong MCD signals, clearly visible in Fig. 1B (solid line). Despite its strong absorption, chl does not contribute to the MCD in this region.

Low-spin oxidized haem gives rise to a strong MCD C-term at 420 nm, along with a series of weaker features reaching above 600 nm. C-terms are paramagnetic in origin, and are distinguished by strong temperature dependence. The 50-K MCD (Fig. 1B, dashed line) identifies the cyt C-term from other absorptions. The derivative-shaped MCD A-term associated with reduced, low-spin haem around 550 nm is unaffected by temperature, as is the weak chl B-term at 670 nm. The A-term of reduced cyt $f$ is shown in detail in Fig. 2 (thick, solid lines). The positive and negative components of the A-term establish the exact wavelengths of the Q transitions, and
The thin, solid lines in Fig. 2 show the green spectral region in detail. MCD signal characteristic of high-spin, ferric haem has appeared. oxidized cyt C-term. At 430 nm however, a temperature-dependent dithionite. Under these conditions, there is no sign of the low-spin, be assigned to a superposition of reduced haems samples. Additional peaks are seen at 556.6 and 561.8 nm which can signal. and asymmetric, supporting the conclusion that this is a composite et al 2000). The MCD A-term accompanying these peaks is broad compared with the absorption.

Figure 1: (A) 1.7-K absorption spectrum of the cyt b6f complex. (B) The corresponding MCD at 1.7 K (solid) and 50 K (dashed). (C) 1.7-K absorption, 1.7-K and 50-K MCD of fully reduced cyt b6f. The data are plotted to equal chl Qy intensities in all samples, for quantitative comparison. The MCD data are magnified 3 times compared with the absorption.

the vibrational side-bands are clearer in the MCD than in the absorption.

MCD Spectra of Fully Reduced and Fully Oxidized cyt b6f. Fully reduced cyt b6f. Figure 1C shows the 1.7-K absorption (thick, solid line) and MCD (thin solid and dotted lines) of cyt b6f reduced by dithionite. Under these conditions, there is no sign of the low-spin, oxidized cyt C-term. At 430 nm however, a temperature-dependent MCD signal characteristic of high-spin, ferric haem has appeared. The thin, solid lines in Fig. 2 show the green spectral region in detail. Features from reduced cyt f are similar to those in the untreated samples. Additional peaks are seen at 556.6 and 561.8 nm which can be assigned to a superposition of reduced haems bH and bL (Schoepp et al 2000). The MCD A-term accompanying these peaks is broad and asymmetric, supporting the conclusion that this is a composite signal.

Fully oxidized cyt b6f. The dashed line in Fig. 2B shows the MCD of cyt b6f under oxidizing conditions. All signs of reduced haem are gone, while a strong C-term from oxidized haem is present. There are no discernible absorption features in this region (not shown).

Quantitative Analysis of the Haem Content of each cyt b6f Sample. Cytochromes give rise to clearly distinguishable and quantitative MCD signals in the ferrous low-spin, ferrous high-spin, and ferric low-spin states. Ferric high-spin haem also gives rise to a distinctive MCD signature, but this signal is too small to be detected among other haem signals. The various forms of cytochrome MCD have been comprehensively reviewed by Cheesman et al (1991).

In the periodate-treated sample (Fig. 2B, dashed line), 100% of the low-spin population is oxidized, as seen by the absence of A-terms around 550 nm and the presence of a strong C-term in the same region. Using this C-term to quantify the C-term present in the non-treated sample (thick, solid line), we find that 60% of the low-spin population is oxidized in this sample. From the location of the single A-term in the non-treated sample, we conclude that the remaining 40% of low-spin haem is reduced cyt f.

In the dithionite-treated sample, there is no oxidized low-spin haem. The cyt f A-term at 550 nm was not increased by the treatment, indicating that it was fully reduced in the untreated sample. The magnitude of the cyt b absorption compared to cyt f indicates that cyt bH and bL are fully reduced (Schoepp et al 2000) under these conditions.

In the present report, we demonstrate the presence of high-spin ferrous haem in cyt b6f under reducing conditions (Fig. 1C). We assign this to cyt c1. Consistent quantification of the ferric cyt MCD signals requires that ferric cyt c1 is high-spin as well. Comparing the high-spin, reduced cyt MCD signal at 430 nm (Fig. 1C) to the literature (Cheesman et al 1991), it appears about 50% as strong as expected. This low intensity could mean that only half of the cyt c1 population is reduced by dithionite, leaving half in the (not discernible) ferric high-spin state. This would require quite a low redox potential of cyt c1, which is perhaps less likely, seeing that it is positioned next to the inner quinone binding site and is therefore likely to undergo frequent redox chemistry. Alternatively, the nature of cyt c1 could be such that the ferrous MCD signal is inherently weak. This would be expected if the spin-orbit coupling of the excited states is low (Cheesman et al 1991).
CONCLUSIONS
Our MCD data from the cyt $b$,$c$ complex under different conditions are consistent with cyt $c_1$ being in a high-spin configuration and cyt $f$, $b_{11}$ and $b_1$ in low-spin configurations, irrespective of redox state.

In the untreated sample, cyt $f$ is fully reduced (low-spin), cyt $b_L$ and cyt $b_{11}$ are fully oxidized (low-spin), as is cyt $c_1$ (high-spin). Periodate fully oxidizes cyt $f$, cyt $b_{11}$ and cyt $b_1$ (low-spin), as well as cyt $c_1$ (high-spin). Dithionite reduces cyt $f$, cyt $b_{11}$ and cyt $b_1$ fully (low-spin), and reduces cyt $c_1$ (high-spin) to 50% or more.

We believe cyt $c_1$ to be fully reduced by dithionite, but with inherently weak MCD, indicating weak spin-orbit coupling between the excited states.

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REFERENCES

ADDITION OF THE Q$_i$ SITE INHIBITOR ANTIMYCIN CHANGES THE ENVIRONMENT OF THE 2Fe2S CLUSTER AT THE Q$_o$ SITE OF THE CYT bc$_1$
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INTRODUCTION
Most of the bacterial ubiquinol (UQH$_2$): cytochrome (cyt) c oxidoreductases (cyt bc$_1$) contain three subunits (cyt $b$, cyt $c_1$ and the FeS protein) carrying four prosthetic groups ($b$-high, $b$-low and $c_1$ hemes and a 2Fe2S cluster, respectively), and form two active sites, Q$_o$ (UQH$_2$ oxidation) and Q$_i$ (UQ reduction) on the p and n sides of the membrane. A unique aspect of Q$_o$ site catalysis lies in the fact that the FeS subunit initiates a bifurcated oxidation of the UQH$_2$, with the first electron being conveyed to the 2Fe2S cluster, which “shuttles” its electron via a rotational displacement from the cyt $b$ to the cyt $c_1$, and the other to the $b$-low heme (Trumpower et al 1978, Crofts et al 1999). The movement can be divided into two portions, a micro- and a macro-movement, reflecting the oscillations of the 2Fe2S at the cyt $b$ surface and its swing to near the cyt $c_1$, respectively. Whether the Fe-S movement is a simple tethered diffusion, or is tightly regulated to insure correct Q$_o$ site turnover remains unclear. Of note, is that when electron transfer through the $b$ hemes to the UQ at the Q$_i$ site is blocked by the addition of Q$_i$ site inhibitors such as antimycin, the electrons derived from the UQH$_2$ oxidation at the Q$_o$ site do not leak back into the chain consisting of electron transfer through the 2Fe2S to the $c_1$ heme (Gray et al 1994, Crofts et al 1999). However, under these conditions, the Fe-S may retain the ability to oxidize UQH$_2$ at the Q$_o$ site via possible by-pass reactions, but only at extremely low rates (~1–2%) compared to uninhibited turnover (Muller et al 2001).

Currently, how such a strict control is achieved during the multiple turnovers of the Q$_o$ site oxidation reactions is unclear, and many proposals attempt to address this issue. A possibility would be to limit the ability of the 2Fe2S cluster to return to the Q$_o$ site when not appropriate (e.g. in the low potential pathway there is no thermodynamically stable repository for the second electron derived from UQH$_2$ oxidation). If and how this may be accomplished remains an open question. We present here the first spectral evidence documenting that the events at the Q$_i$ site affect the steady state interactions of the 2Fe2S with the occupant of the Q$_o$ site. These interactions appear to be dependent on: the presence of the antimycin at the Q$_i$ site, the ability of the Fe-S subunit to undergo a micro-motion, and on the Q/QH$_2$ occupancy of the Q$_o$ site of the cyt bc$_1$.

MATERIALS AND METHODS
Bacterial strains and growth conditions. All R. capsulatus strains, their construction and phenotypes are as described previously (Darrouzet et al 2000).

Preparation and spectroscopic analysis of ordered membrane sample. Membrane isolation and ordered sample preparation is described in detail in Cooley et al 2004. Antimycin and HQNO concentrations were approximately 10 and 30 μM per ~30 mg/ml total membrane protein, respectively. Chemical reduction was by addition 5 mM Na-Ascorbate.

RESULTS/DISCUSSION
Competitive Inhibition at the Q$_i$ Site by Antimycin Alters the EPR Spectral Shape of Ordered Membrane Samples. To address the possibility that antimycin causes an alteration of the steady-state position of the 2Fe2S (i.e. less Fe-S at the cyt $b$ Q$_o$ site), ordered membrane samples from wild type (WT) R. capsulatus membranex and a variety of hinge region strains, the +nAla mutants (+1Ala, +2Ala, +3Ala; known to be slowed in the macro-movement, deficient in the macro-movement, or devoid of both the macro- and micro-movements of the Fe-S, respectively (Darrouzet et al 2000, Cooley et al 2004) were treated with antimycin and subjected to oriented EPR analysis. Oriented EPR analysis yields specific information about the relative orientation (and relative numbers of different orientations) of the [2Fe2S] cluster in a given sample, as well as increased spectral resolution of a given transition in the EPR spectrum.