# Communication

## Characterization of DorC from *Rhodobacter capsulatus*, a *c*-type Cytochrome Involved in Electron Transfer to Dimethyl Sulfoxide Reductase*

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The *dorC* gene of the dimethyl sulfoxide respiratory (*dor*) operon of *Rhodobacter capsulatus* encodes a pentaheme *c*-type cytochrome that is involved in electron transfer from ubiquinol to periplasmic dimethyl sulfoxide reductase. DorC was expressed as a C-terminal fusion to an 8-amino acid FLAG epitope and was purified from detergent-solubilized membranes by ion exchange chromatography and immunoaffinity chromatography. The DorC protein had a subunit $M_r = 46,000$, and pyridine hemochrome analysis indicated that it contained 5 mol heme c/mol DorC polypeptide, as predicted from the derived amino acid sequence of the *dorC* gene. The reduced form of DorC exhibited visible absorption maxima at 551.5 nm (α-band), 522 nm (β-band), and 419 nm (Soret band). Redox potentiometry of the heme centers of DorC identified five components ($n = 1$) with midpoint potentials of −34, −128, −184, −185, and −276 mV. Despite the low redox potentials of the heme centers, DorC was reduced by duroquinol and was oxidized by dimethyl sulfoxide reductase.

The ability to use Me₂SO and trimethylamine-*N*-oxide (TMAO)[1] as electron acceptors is widespread among facultative aerobic bacteria and the organization of the Me₂SO and TMAO respiratory chains is now well defined (1). In the Me₂SO respiratory system of purple photosynthetic bacteria such as *Rhodobacter capsulatus*, electrons are transferred from primary dehydrogenases via the ubiquinone pool to a periplasmic Me₂SO reductase (2). Recent sequence and mutational analysis of the Me₂SO respiratory gene cluster from photosynthetic bacteria has identified a pentaheme *c*-type cytochrome (DorC)

as the likely mediator of electron transfer from ubiquinol to Me₂SO reductase (3).[2] The TMAO respiratory (Tor) system of *Escherichia coli* (5) is very similar to the Dor system of *R. capsulatus*, and they differ from the Me₂SO respiratory (Dms) system of *E. coli*. The Me₂SO reductase (DmsABC) of *E. coli* can be purified as a menaquinol-oxidizing Me₂SO reductase complex that lacks *c*-type cytochromes (6). Me₂SO reductase from *R. capsulatus* has been purified as a monomeric protein containing a pterin molybdenum cofactor (Moco) as its only prosthetic group (7). This property of Me₂SO reductase has many advantages for spectroscopic characterization of Moco, but a major deficiency has been the lack of a physiologically relevant electron donor. To overcome this problem we describe in this paper the purification and characterization of DorC.

The derived amino acid sequence of *Rhodobacter* DorC indicated that it was related to members of the NirT class of tetraheme *c*-type cytochromes (3).[2] However, DorC is predicted to be a pentaheme cytochrome with a fifth *c*-type heme binding motif in the C-terminal polypeptide, which is absent from the tetraheme members of the NirT class. Almost all of the members of the NirT class are involved in an anaerobic respiratory pathway, and their role appears to be to catalyze electron transfer from the Q-pool to a periplasmic terminal reductase (8). Although molecular genetic studies have provided an insight into the function and likely properties of cytochromes of the NirT class, very little is known about their biochemical and spectroscopic properties.

## EXPERIMENTAL PROCEDURES

*Expression and Purification of DorC*—A 1.4-kilobase DNA fragment containing the complete *dorC* nucleotide sequence except for the stop codon and including the upstream regulatory sequences of the *dor* operon was amplified by polymerase chain reaction using plasmid pALS3 as a template.[2] The forward primer was designed so that a *Bam*HI site would be synthesized while the reverse primer directed the synthesis of an *Eco*RI site plus a sequence to include a C-terminal 8-amino acid FLAG epitope (9). The 1.4-kilobase polymerase chain reaction product was cloned into pDSK519 (10) to create plasmid pALS300. pALS300 was transferred from *E. coli* S17–1 to *R. capsulatus* 37b4 by conjugation (11).

DorC was expressed in 2l *R. capsulatus* cells grown for 24 h under phototrophic conditions in RCV medium (12) in the presence of 60 mM Me₂SO. Membranes were prepared from the cells using a French Press as in Ref. 2 and were resuspended in Tris-buffered saline. Proteins were solubilized by treatment of membranes with 4% Triton X-100 for 90 min at room temperature. The mixture was then centrifuged ($145,000 \times g$, 90 min, 4 °C), and the soluble fraction was collected. The soluble fraction containing DorC (containing approximately 100 mg of protein) was dialyzed against 50 mM Tris-HCl, pH 8.0, 0.2% Triton X-100 and then charged onto a DEAE-Trisacryl column (bed volume, 15 ml), equilibrated with the same buffer. DorC was eluted using a 100-ml linear gradient of NaCl from 0 to 1 M. The DorC-enriched fraction from the anion exchange chromatography was dialyzed against Tris-buffered saline plus 0.2% Triton X-100 and then charged onto a 2-ml column containing FLAG affinity resin (Eastman Kodak). Bound DorC was eluted with 0.1 M glycine, pH 3.5, 0.2% Triton X-100. 2-ml fractions were collected, and the pH was immediately raised by addition of 50 μl 1 M Tris-HCl, pH 8.0.

*Spectroscopy and Redox Potentiometry*—Optical spectra of DorC were collected using a Hitachi U-3000 spectrophotometer. Pyridine hemochrome analysis of DorC was carried out according to the method of Berry and Trumpower (13). For reduction of DorC with duroquinol, the cytochrome was incubated with 2 units ml⁻¹ Clostridial diaphorase,

[1] The abbreviations used are: TMAO, trimethylamine-*N*-oxide; PAGE, polyacrylamide gel electrophoresis.

[2] Shaw, A. L., Knaeblein, J., Leimkuhler, S., Hanson, G. R., Klipp, W., and McEwan, A. G. (1999) *Microbiology*, in press.
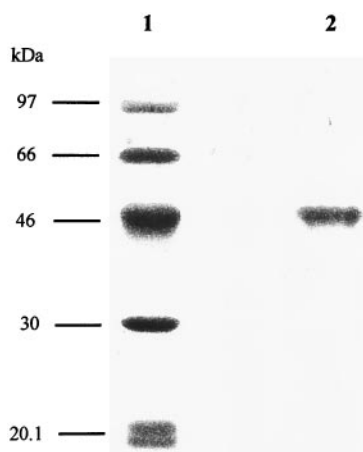
FIG. 1. **Coomassie-stained SDS-PAGE gel of purified DorC.** *Lane 1*, molecular mass markers; *lane 2*, 8 $\mu$g of purified DorC.
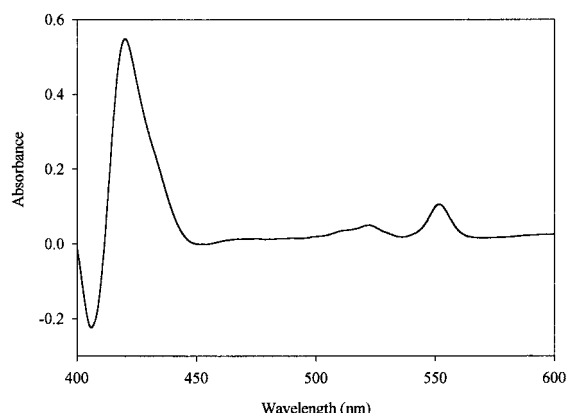


FIG. 2. **Dithionite reduced minus ferricyanide oxidized difference spectrum of DorC.**



FIG. 3. **Pyridine hemochrome (*A*) and pyridine hemichrome (*B*) spectra of DorC.**



FIG. 4. **Redox potentiometry of DorC (12 $\mu$M).** Symbols represent the results of two separate titrations. The *solid line* is a theoretical curve generated using the Nernst equation for five $n = 1$ oxidation-reduction processes with midpoint redox potentials $-34$, $-128$, $-184$, $-185$, and $-276$ mV with relative contributions to the total absorbance signal of 10, 22, 15, 30, and 23%, respectively.

25 $\mu$M duroquinone and 200 $\mu$M NADH. Redox potentiometry of DorC was performed by the method of Dutton (14). Absorption spectra were recorded between 500 and 600 nm using a Jasco 7850 UV-visible spectrophotometer. Redox titrations were performed with dithionite as reductant and ferricyanide as oxidant in the presence of the following mediators: 2.5 $\mu$M benzoquinone (Eo' = +280 mV), 2.5 $\mu$M 1,2-naphthoquinone (Eo' = +145 mV), 2.5 $\mu$M 1,4-naphthoquinone (Eo' = +60 mV), 20 $\mu$M duroquinone (Eo' = +5 mV), 20 $\mu$M 1,4-dihydroxynaphthoquinone (Eo' = −145 mV), 5 $\mu$M anthroquinone-2-sulfonate (Eo' = −225 mV), and 5 $\mu$M benzylviologen (Eo' = −350 mV). The absorbance change at 551.5–540 nm was plotted against redox potential, and theoretical Nernstian curves were fitted to the data using Sigma Plot.

*Protein Methods*—SDS-PAGE analysis of proteins and Western blotting using an anti-FLAG antibody were performed as in Refs. 15 and 16. Protein concentration was determined as in Ref. 17.

<div align="center">RESULTS</div>

*Expression and Purification of DorC*—A plasmid for the expression of DorC with an 8-amino acid FLAG epitope attached to the C terminus was constructed as described under "Experimental Procedures." This plasmid was expressed in *R. capsulatus* cells grown phototrophically in the presence of Me₂SO. Membrane and soluble fractions were prepared, and production of DorC was monitored by Western blotting. A single immunoreactive polypeptide with a molecular mass of 46 kDa was observed; this was the expected size of the DorC-FLAG polypeptide. The putative DorC polypeptide was located exclusively in the membrane fraction. Several detergents were tested for their ability to solubilize DorC. Triton X-100 proved to be the most efficient, but even this detergent failed to completely solubilize DorC because an immunoreactive polypeptide could still be detected in the detergent-insol-
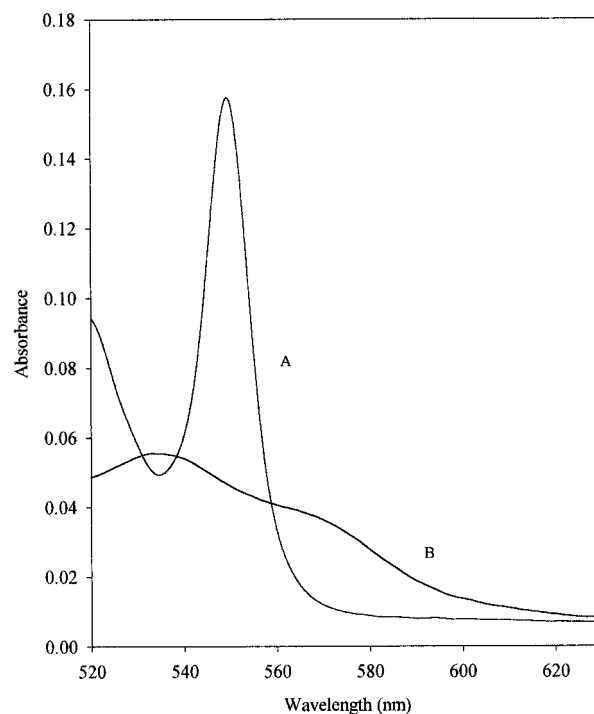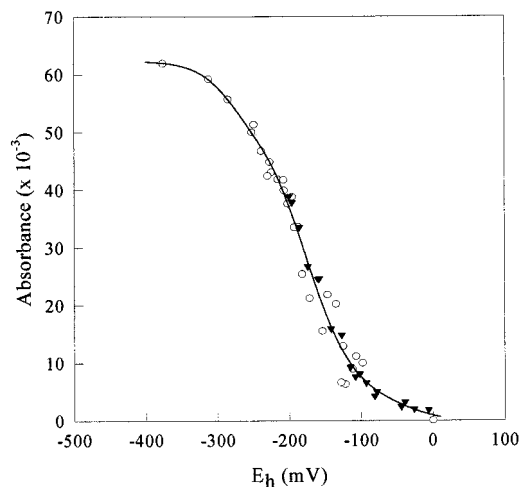
uble membrane fraction.

The detergent-solubilized fraction was applied to a DEAE-Trisacryl anion exchange column. DorC was eluted from the column at about 450 mM NaCl. The fractions containing DorC were then applied to a FLAG-affinity resin and eluted as described under "Experimental Procedures." The pooled fractions containing the colored cytochrome were analyzed by SDS-PAGE. Fig. 1 shows the presence of a single polypeptide $M_r = 46,000$ corresponding to DorC. This protein cross-reacted with the anti-FLAG antibody in Western blots and stained for heme-dependent peroxidase activity (data not shown), confirming that the DorC-FLAG polypeptide had been purified.

*Optical Spectroscopy of DorC*—Fig. 2 shows a dithionite-reduced minus ferricyanide-oxidized spectrum of DorC. Absorption peaks at 551.5 nm ($\alpha$-band), 522 nm ($\beta$-band), and 419
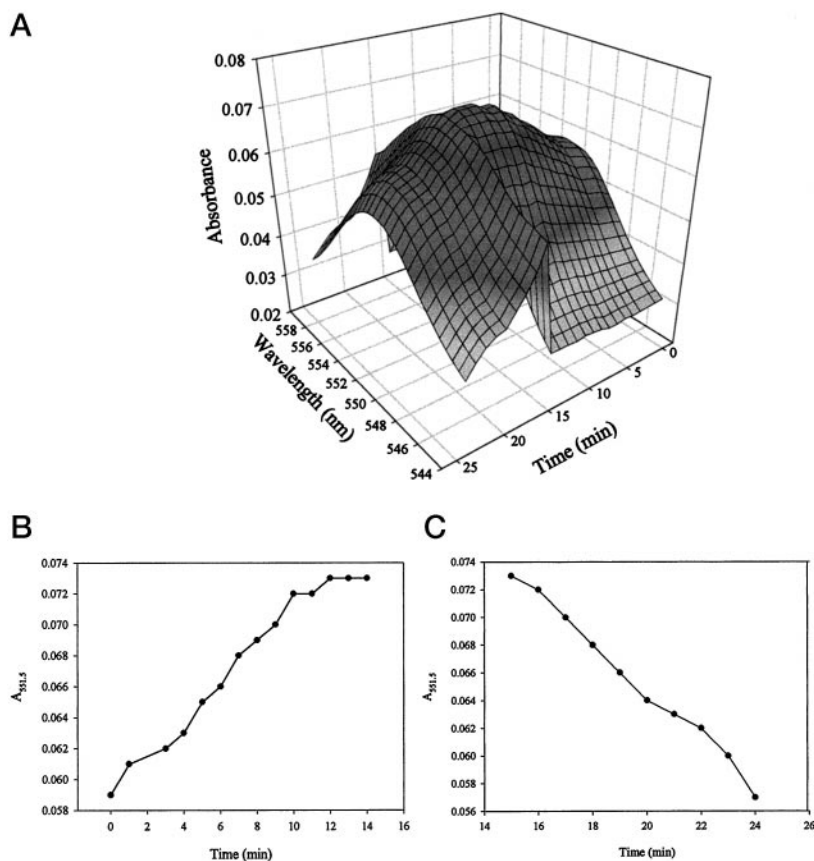
FIG. 5. *A*, increase in the absorbance of the α-band of DorC with time (0–14 min) upon incubation of 8.0 $\mu$M DorC in 20 mM HEPES-NaOH, pH 7.2, 1 mM EDTA, 0.1% Triton X-100 with 2 units ml$^{-1}$ diaphorase, 20 $\mu$M NADH, and 10 $\mu$M duroquinone. At time 15 min, 3 $\mu$M Me$_2$SO reductase was added to the reduced DorC, and the decrease in the absorbance of the α-band was measured from time 16 min. *B*, increase in absorbance at 551.5 nm against time. *C*, decrease in absorbance at 551.5 nm against time.

nm (Soret band) were typical of a reduced cytochrome. The absorption minimum at 408 nm represents the Soret absorption band of the oxidized form of DorC. A spectrum of the resting form of DorC exhibited this 408 nm peak, and the absence of the α-band and β-band of the reduced cytochrome indicates that the DorC, as prepared, is in a fully oxidized state.

The number of heme groups per DorC polypeptide was determined by pyridine hemochrome analysis. Fig. 3 shows hemichrome and hemochrome spectra of DorC in alkaline pyridine. The presence of an absorption maximum at 550 nm in the pyridine hemochrome spectrum (Fig. 3) confirmed that DorC contained only *c*-type hemes. Using the method of Berry and Trumpower (13), it was calculated that there were 4.9 mol *c*-type heme/mol DorC. This confirms that DorC is a pentaheme, as indicated from the primary structure analysis.[2]

*Determination of the Midpoint Redox Potential of the Heme Centers of DorC*—The thermodynamic properties of the heme centers in DorC were analyzed by redox potentiometry. It was observed that during reductive titration no reduction of the cytochrome was seen until the ambient redox potential (at pH 7.0) was below 0 mV. This is consistent with the observation that sodium ascorbate ($E_m$ = +65 mV) did not reduce DorC as seen in reduced *minus* oxidized difference spectra (data not shown). Fig. 4 shows the change in absorbance of the a band (551.5 nm minus 540 nm as the reference wavelength) of DorC as a function of the ambient redox potential. The data obtained could be fitted to a five-component Nernstian curve ($n$ = 1) with midpoint potentials ($E_{m7.0}$) of $-34 \pm 2$, $-128 \pm 2$, $-184 \pm 3$, $-185 \pm 3$, and $-276 \pm 3$ mV.

*DorC-dependent Electron Transfer from Duroquinol to Me$_2$SO Reductase*—DorC is the only protein encoded by the *dor* operon that could act as a mediator of electron transfer between ubiquinol and Me$_2$SO reductase.[2] Before reconstitution of this electron transfer pathway was attempted, the ability of

duroquinol to reduce DorC was investigated. A duroquinol-reduced spectrum of DorC indicated that at least some of the heme centers of the protein had been reduced (data not shown).

For the reconstitution of electron transfer from quinol to Me$_2$SO reductase DorC was titrated with an approximately stoichiometric amount of duroquinol under anaerobic conditions. This led to an increase in absorbance at 551.5 nm (Fig. 5). Upon addition of Me$_2$SO reductase there was a decrease in the absorption of the α-band of DorC (Fig. 5), indicating that electron transfer from DorC to Me$_2$SO reductase had occurred. Control experiments showed that the oxidation of DorC was specifically dependent upon Me$_2$SO reductase.

### DISCUSSION

DorC from *R. capsulatus* is the first pentaheme cytochrome in the NirT class to be purified and characterized. The presence of photosynthetic pigments in the membrane of *R. capsulatus* made the detection of the native cytochrome very difficult and necessitated the strategy of attaching a FLAG peptide as a tag for detection and affinity purification. The protein was purified from *R. capsulatus* because all of our attempts to express DorC in *E. coli* were unsuccessful.[3] DorC was located exclusively in the membrane of *R. capsulatus*, and this would be consistent with a role in ubiquinol oxidation. *In vivo,* it is expected that DorC would be a peripheral membrane protein anchored to the membrane by its hydrophobic N terminus, and this explains why detergents were required to solubilize the protein.

The optical spectroscopy of DorC identified an absorption maximum at 551.5 nm for the α-band. This is a symmetrical peak with very little complexity, and it suggests that although the midpoint redox potentials of the heme centers differ, it is likely that each possesses the same fifth and sixth ligands. There was no evidence for high spin five coordinate heme with

[3] A. L. Shaw and A. G. McEwan, unpublished observations.

an optical band beyond 600 nm as seen in cytochrome *c'* for example (18). Redox potentiometry was consistent with the presence five heme centers, all of which have midpoint redox potentials below 0 mV. These are surprisingly low values for a protein that is thought to act as an electron acceptor from ubiquinol because the Eo′ for the ubiquinol/ubiquinone redox couple is +40 mV (19). However, it was observed that water-soluble duroquinol could reduce DorC. The thermodynamic quantity described in this paper is the "macroscopic" potential of the heme centers of DorC (20, 21). These parameters are not influenced by electron/electron cooperativity between heme centers. However, it has been shown that in the tetraheme cytochrome $c_3$ from *Desulfovibrio vulgaris* each heme redox potential is dependent on the oxidation state of the other three hemes (22), and this can affect the thermodynamic properties of a multi-heme protein (21). This can lead to a variety of potential microscopic heme-heme interacting potentials that may be higher than the macroscopic potentials. The ability of DorC to be reduced by duroquinol can be rationalized if the heme with the highest potential ($-34$ mV) is reducible by duroquinol. Under physiological conditions this would suggest that the DorC protein would be reduced when the $E_h$ of the ubiquinol/ubiquinone pool was lower than the Eo′ for the ubiquinol/ubiquinone redox couple. Such a situation could occur under phototrophic (anaerobic) conditions in the presence of reduced carbon sources (23). Further work to demonstrate and analyze heme-heme interactions in DorC using EPR and/or NMR spectroscopy is now required.

There has been intense interest in the structure and mechanism of $Me_2SO$ reductase (4, 24, 25), and many questions remain regarding the route of electron transfer to the molybdenum atom. It seems almost certain that one of the molybdopterin guanine dinucleotide moieties of Moco will act as a conduit for electrons from DorC. The purification of DorC that we have described opens the way for experiments to look at electron transfer to $Me_2SO$ reductase using the physiological electron donor. This may help resolve some current controversies relating to the mechanism of $Me_2SO$ reductase by avoiding the need to use dithionite and viologens as electron donors. Although we showed that DorC was reduced by duroquinol and that reduced DorC was oxidized by $Me_2SO$ reductase, it was not possible to use DorC to catalyze steady state electron transfer between duroquinol and $Me_2SO$ reductase. The reason is

that $Me_2SO$ reductase itself exhibited duroquinol-$Me_2SO$ oxidoreductase activity.[3] However, under physiological conditions DorC would be required for the oxidation of ubiquinol-10, which, unlike water-soluble duroquinol, is buried within the hydrophobic domain of the cytoplasmic membrane and thus is inaccessible to periplasmic $Me_2SO$ reductase.

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