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Electron-Paramagnetic-Resonance Spectroscopy of *Bacillus subtilis* Cytochrome b_{558} in *Escherichia coli* Membranes and in Succinate Dehydrogenase Complex from *Bacillus subtilis* Membranes

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Cytochrome b_{558} of the *Bacillus subtilis* succinate dehydrogenase complex was studied by electron-paramagnetic-resonance (EPR) spectroscopy. The cytochrome amplified in *Escherichia coli* membranes by expression of the cloned cytochrome gene and in the succinate dehydrogenase complex immunoprecipitated from solubilized *B. subtilis* membranes, respectively, is shown to be low spin with a highly anisotropic (g_{max} = 3.5) EPR signal. The amino acid residues most likely forming fifth and sixth axial ligands to heme in cytochrome b_{558} are discussed on the basis of the EPR signal and the recently determined gene sequence (K. Magnusson, M. Philips, J. R. Guest, and L. Rutberg, J. Bacteriol. 166:1067–1071, 1986) and in comparison with other *b*-type cytochromes.

Cytochrome b_{558} in the aerobic gram-positive bacterium *Bacillus subtilis* is a transmembrane protein that specifically anchors succinate dehydrogenase (SDH) to the inner surface of the cytoplasmic membrane (16, 17). The *B. subtilis* SDH-cytochrome b_{558} complex is analogous to the mitochondrial succinate-Q reductase complex (complex II) (10) but differs from it in that the bacterial cytochrome is rapidly reduced by succinate in membranes, as well as in the isolated SDH complex (11). The difference of the reduced minus the oxidized light absorption spectrum of the cytochrome exhibits maxima at 426, 529, and 558 nm at room temperature.

The apocytochrome is coded for by the *B. subtilis sdhA* gene, which has been cloned (21) and sequenced (22). It is, with the exception of the *Escherichia coli sdhC* and *sdhD* genes (7), the only cytochrome *b* of a complex II for which the sequence is known. The cloned *sdhA* gene can be expressed in *E. coli* cells, resulting in a membrane-bound cytochrome b_{558} (21). To our knowledge, it is the first example reported of a *b*-type cytochrome that has been expressed in a foreign host. The cytochrome synthesized in *B. subtilis* and *E. coli* shows identical light absorption difference spectra, and the respective proteins have the same electrophoretic mobilities, m_r 19,000, in sodium dodecyl sulfate-polyacrylamide gels (21). The molecular weight, deduced from the nucleotide sequence, is 22,770 (22).

In the present work, we used electron-paramagnetic-resonance (EPR) spectroscopy to study the *B. subtilis* cytochrome b_{558} both in *E. coli* membranes and in the SDH complex isolated from *B. subtilis* membranes. This was done to compare the EPR signal of the hemoprotein at the two locations and to obtain information on the coordination environment of the heme iron in cytochrome b_{558}. The heme prosthetic group in cytochrome b_{558} is required for the tight and functional binding of *B. subtilis* SDH to the cytochrome (17). In the absence of heme, soluble SDH subunits accumulate in the cytosol (15), whereas apocytochrome is incorporated into the cytoplasmic membrane (H. Fridén and L. Hederstedt, unpublished data). Considering the crucial role of heme in the assembly of the complex and in the function of cytochrome b_{558} as electron acceptor from SDH, it was also interesting to determine whether the EPR spectrum of the heme is altered when SDH is bound.

Wide-scan EPR spectra of *B. subtilis* SDH-cytochrome b_{558} complex. The SDH-cytochrome b_{558} complex was isolated from Triton X-100-solubilized *B. subtilis* 168 cytoplasmic membranes by immunoprecipitation with anti-SDH antiserum. The precipitate contained, in addition to immunoglobulins, three polypeptides in equimolar amounts, i.e., the two SDH subunits and cytochrome b_{558} (12). EPR spectra of the immunoprecipitated complex recorded at 9 K are shown in Fig. 1. Signals can be detected at *g* values of about 6, 4.2, 3.5, and 2 in the spectrum of the oxidized sample (Fig. 1A). The signal at a *g* value of 2.02 is from iron-sulfur cluster S-3 of SDH (13). The signal at a *g* value of around 4.2 is probably not a ferric heme resonance (for a review on heme EPR signals, see reference 28) and probably represents adventitious iron, which produces an intense EPR signal. The signal at a *g* value of 6 was not seen in all preparations of the complex and probably originates from ferric high-spin (S = 5/2) heme. The broad signal at a *g* value of about 3.5 is similar to the ferric low-spin (S = 1/2) heme resonance reported for cytochrome b_{560} of mitochondrial complex II (26).

High-spin ferric heme generally results in a high amplitude of the first derivative of the EPR signal at a *g* value of 6 compared with that of low-spin heme, especially with g_{max} values above 3.2. From the relative intensities of signals from *g* values equal to approximately 3.5 and 6 (Fig. 1A), the concentration of low-spin heme in the preparation must be more than one magnitude larger than that of the high-spin heme. (For an example of the relative intensities of high- and low-spin ferric heme EPR signals, see Fig. 6 of reference 1.)

Reduction of the immunoprecipitated SDH-cytochrome b_{558} complex with 10 mM succinate (data not shown) or 8 mM dithionite (Fig. 1B) resulted in an almost complete disappearance of the signal with a *g* value of approximately 3.5. In parallel, the EPR signal of cluster S-3 disappeared, and signals at *g* values equal to 2.035, 1.94, and 1.89, originating from iron-sulfur cluster S-1 of SDH, appeared

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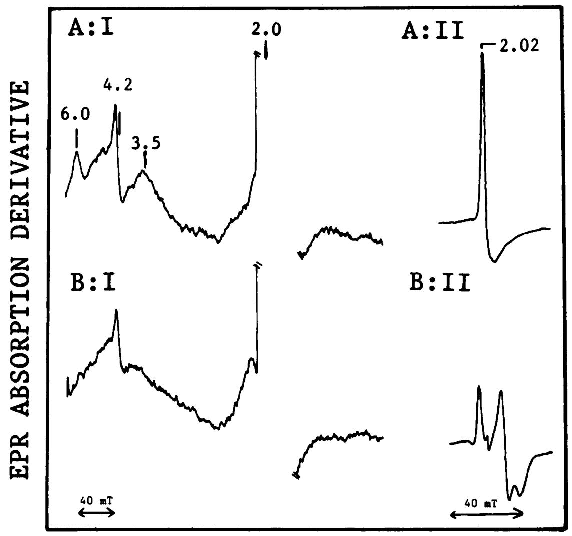

FIG. 1. Wide-scan EPR spectra of immunoprecipitated *B. subtilis* SDH-cytochrome b_{558} complex. Spectrum A, Ferricyanide-oxidized complex. Spectrum B, Dithionite-reduced complex. The *g* values are indicated at the top of the figure. Spectra A:II and B:II show the region with a *g* value of 2 on an expanded scale and recorded at 1/10 the amplification used in A:I and B:I, respectively. The immunoprecipitate isolated as described before (12) was in 10 mM morpholinepropanesulfonic acid (MOPS) buffer (pH 7.4). Cryogenic spectroscopy was done at X-band frequency on a Varian E-109 spectrometer equipped with an Oxford Instruments helium temperature-flow cryostat system. Spectra were digitized by an 8-byte Cromenco microcomputer interfaced to the spectrometer. Datum points were processed with a program made by T. Astlind. The magnetic field was determined as proton nuclear resonance with an AEG Kernresonanz-Magnetfeldmesser. EPR conditions were as follows: sample temperature, 9 K; microwave frequency, 9.1 GHz; microwave power, 4 mW; modulation amplitude, 1 mT; time constant, 1 s; scanning rate, 6.25 mT/min. The concentration of SDH complex was 26 μM, as determined (11) from the covalently bound flavin.

(13). These findings indicate that the resonance at a *g* value of about 3.5 is from cytochrome b_{558}.

EPR signals from cytochrome in *B. subtilis* membranes. *B. subtilis* membranes can contain cytochromes of the types aa_3, *b*, *c*, and *o* (34, 35). The types and relative amounts vary with strains and growth conditions. In the membranes used in this work, cytochrome b_{558}, when present, is the most abundant cytochrome, as judged by light absorption spectroscopy of isolated membranes (11, 14). EPR spectra of oxidized membranes from two *B. subtilis sdh* mutants, KA98011 (*trpC2 sdhB11*) (14) and KA97115 (*trpC2 leu-2 sdh-115*) (23) are shown in Fig. 2. Both mutants lack membrane-bound SDH. Mutant KA98011 contains normal amounts of cytochrome b_{558}, but KA97115 specifically lacks this cytochrome. Significant signals attributable to cytochrome b_{558} could not be seen in the spectra of highly concentrated KA98011 or wild-type membranes (the entire 4.2 to 77 K temperature interval was analyzed). The EPR measurements were sensitive enough, however, to detect in both mutant membranes signals from copper atoms and from a trace of low-spin ferric heme with a g_{max} value of 3, probably from cytochrome *a* or *o*. These results with membranes agree with the very low intensity of the heme signal with a *g* value of approximately 3.5 detected in the isolated SDH complex and the small amount (about 0.5% of the membrane protein) of cytochrome b_{558} in *B. subtilis* wild-type and KA98011 membranes.

EPR spectra of *B. subtilis* cytochrome b_{558} in *E. coli* membranes. Large amounts of membrane-bound cytochrome b_{558} (5% of the total membrane protein) can be produced in *E. coli* by expression of the cloned *B. subtilis sdhA* gene on a multicopy plasmid (21). EPR spectra at 10 K of membranes prepared from *E. coli* 5K cells containing plasmid pKIM2 or pKIM6 are shown in Fig. 3. Plasmid pKIM2 carries the *sdhA* gene, whereas pKIM6 is the same vector with an insert of *B. subtilis* DNA from outside the *sdh* operon (21). In the EPR spectrum of oxidized *E. coli* 5K(pKIM2) membranes (Fig. 3A), a signal at a *g* value of 3.47 ± 0.05 was observed similar to that found for the *B. subtilis* SDH-cytochrome b_{558} complex. This signal was not seen in oxidized membranes from *E. coli* 5K(pKIM6) (Fig. 3D). The small signal(s) in the region with a *g* value of 3.5 that can be seen in Fig. 3D is from *E. coli* 5K cytochromes.

The signal with a *g* value of approximately 3.5 from *E. coli* 5K(pKIM2) membranes decreased in intensity when the preparation was reduced at room temperature for 5 min with 5 mM ascorbate and 10 μM *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (spectrum not shown), in accordance with the partial reduction of cytochrome b_{558} by this treatment, as determined by light absorption spectroscopy. Addition of dithionite (pH 7.5) and incubation at room temperature for 1 min caused complete reduction of cytochrome b_{558} and disappearance of the EPR signal with a *g* value of approximately 3.5 (Fig. 3B). The difference of oxidized minus reduced EPR spectrum of *E. coli* 5K(pKIM2) membranes (Fig. 3C) shows the signal with the *g* value of about 3.5 more clearly, and there is a small resonance with a *g* value of 6 and

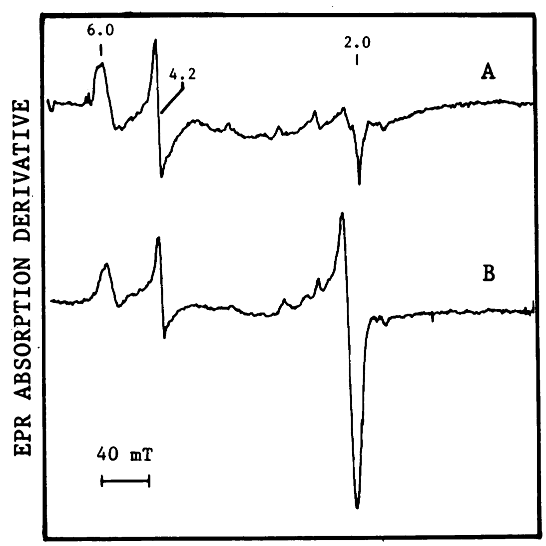

FIG. 2. Wide-scan EPR spectra of membranes from two *B. subtilis* mutants. Spectrum A, Air-oxidized membranes from mutant KA98011 which contain normal amounts of cytochrome b_{558}. Spectrum B, Air-oxidized membranes of mutant KA97115 which lack cytochrome b_{558}. The spectra shown are of four accumulated repetitive scans. The bacteria were grown in complex medium, and membranes were isolated as described previously (13) and stored at −80°C in 20 mM MOPS buffer (pH 7.4). The membrane protein concentrations were 46 mg/ml, as determined by the procedure of Lowry et al. (20) in the presence of 1.7% (wt/vol) sodium dodecyl sulfate and with bovine serum albumin as the standard. EPR measurements and conditions were as in Fig. 1, except that the sample temperature was 12 K and the microwave power was 1 mW.

signals in the region with a g value of 2 from *E. coli* membrane-bound iron-sulfur clusters (18), mainly in SDH (7). The signal with a g value of 3.5 could be detected also at 27 K but was less pronounced than at 10 K.

We conclude that *B. subtilis* cytochrome b_{558} is of low spin with a g_{max} equal to 3.47 ± 0.05 and that it apparently has an identical EPR spectrum whether present in SDH complex solubilized from *B. subtilis* membranes or in *E. coli* membranes. This finding is of importance since the EPR signal of hemoproteins is sensitive to changes in the close environment of the heme group. For example, the two g_{max} values of cytochrome *b* from yeast ubiquinol-cytochrome *c* reductase (complex III) convert to a resonance with a single g_{max} upon purification of the protein (36), and also, the redox potential and EPR spectrum of chloroplast cytochrome b_{559} are easily altered, as discussed by Babcock et al. (2). *B. subtilis* cytochrome b_{558} in the *E. coli* membranes does not, in contrast to in the *B. subtilis* complex, have SDH bound (21). The results thus indicate that the EPR spectrum of the cytochrome is not affected by the dehydrogenase despite the direct acceptor function of cytochrome b_{558} for electrons from bound SDH.

Axial heme ligands. The EPR spectrum of *B. subtilis* cytochrome b_{558} described here is similar in both shape and g_{max} value to the recently reported highly anisotropic low-spin (HALS) spectra (25, 32). Because of the overlapping signals from iron-sulfur clusters in the region with a g value of 2 and presumably the very broad and low intensity of any g_{min} signals, we could not assign additional EPR features to the g_{max} signal of the cytochrome.

Several membrane-bound *b*-type cytochromes have been reported to show HALS spectra. (i) Cytochrome b_{560} in mitochondrial complex II has a g_{max} of 3.5. The EPR spectrum of oxidized bovine heart complex II (Fig. 1D in reference 26) is strikingly similar to that of the *B. subtilis* SDH complex. (ii) Cytochrome b_{562} (b_K) and b_{566} (b_T) of mammalian complex III show g_{max} values of 3.45 and 3.78, respectively (19, 27, 31). (iii) Cytochrome b_{563} (b_6) of chloroplast thylakoid membranes has a g_{max} of 3.5 (3, 4). Also, cytochrome b_{556} of *Micrococcus luteus* which is immunoprecipitated from solubilized membranes in complex with SDH (8) has tentatively been assigned a high g_{max} value (9). The small HALS-type signal seen in the *E. coli* control membranes (Fig. 3D) could be from the cytochrome *b* which copurifies with *E. coli* SDH (7). It should be noted that not all membrane-bound, low-spin, *b*-type cytochromes show high g_{max} values, as exemplified by chloroplast cytochrome b_{559}, with a g_{max} of 3.1 to 2.9 (2, 24).

The two axial ligands to heme iron in cytochromes with HALS-type spectra have not been demonstrated. The amino acid residues considered as possible ligands are histidine, lysine, and methionine.

Bis-histidine ligation has been suggested from studies on model heme compounds (6, 32) and is supported by the amino acid sequence homologies found in cytochrome *b* of complex III from different organisms and in cytochrome b_{563} from chloroplasts (33, 38). All of these cytochromes contain four invariant histidines in pairs suitable for ligation of two hemes and located in hydrophobic, probably membrane-spanning, protein segments. In addition, the mitochondrial proteins contain conserved, positively charged amino acid residues which are proposed to stabilize propionic acid residues of the heme molecule by forming salt bridges. However, the cytochromes *b* for which bis-histidine ligation has been demonstrated, for example, cytochrome b_5, show EPR spectra with a g_{max} near 3.0 (37). A hypothesis has been

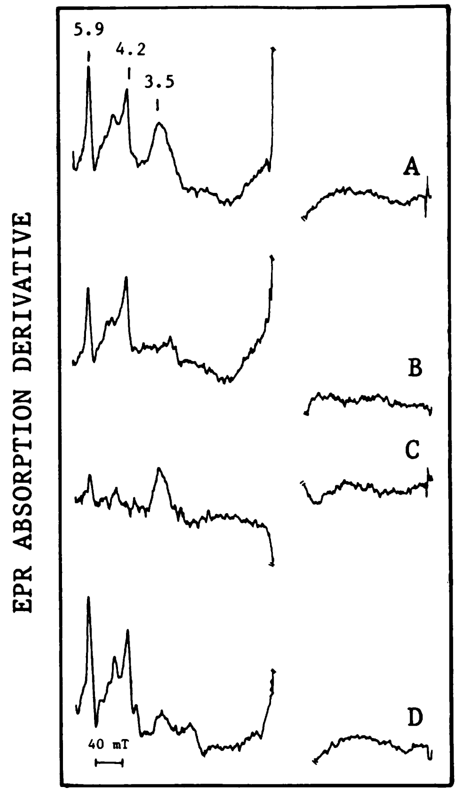

FIG. 3. EPR spectra of *E. coli* membranes containing *B. subtilis* cytochrome b_{558} and of *E. coli* control membranes. Spectrum A, Air-oxidized *E. coli* 5K(pKIM2) membranes which contain *B. subtilis* cytochrome b_{558}. Spectrum B, The same membranes as in spectrum A, but reduced with 8 mM sodium dithionite. Spectrum C, The difference of oxidized minus reduced EPR spectrum resulting from the subtraction of spectrum B from spectrum A. Spectrum D, Air-oxidized *E. coli* 5K(pKIM6) membranes which do not contain the *Bacillus* cytochrome. *E. coli* was grown, and crude membranes were prepared, as described before (21). The concentrations of membrane protein and protoheme for *E. coli* 5K(pKIM2) and 5K(pKIM6) were 61 mg/ml and 98 μM and 77 mg/ml and 15 μM, respectively. Protoheme was determined by the method of Rieske (30), and protein concentrations were determined as described in the legend to Fig. 2. EPR measurements and conditions were as described in the legend to Fig. 1.

developed in which the high g_{max} values of HALS spectra are explained as a result of a "distorted" or "strained" bis-histidine coordination to the heme (6, 29, 32). Palmer (29) has proposed that if the two imidazole-ring planes of the ligating histidines are gradually rotated from a parallel orientation, which gives a g_{max} of about 3.0, towards a perpendicular orientation, the g_{max} value will increase, and the g_{mid} and g_{min} components of the EPR signal will be progressively smaller. An alternative type of coordination that cannot be excluded is heme-iron ligation involving lysine residues. High g_{max} values have been obtained in experiments with amine-heme model complexes and with cytochrome *c* which at an alkaline pH has histidine-lysine ligation (5). Mitochondrial cytochrome c_1 with a g_{max} of 3.35 has, from line-shape analysis of EPR spectra, been speculated to have histidine-methionine axial ligation (31).

The predicted amino acid sequence of *B. subtilis* cytochrome b_{558} contains six histidine, two lysine, and six methionine residues (22). Sequence homologies to other cytochromes *b* have not been found, preventing any conserved residues from being identified. A hydropathy profile of the amino acid sequence indicates five transmembrane segments, four of which contain histidine (at positions 13, 28, 70, 113, and 155) (Fig. 3 in reference 22). Analogous to the complex III-type cytochrome *b*, bis-histidine ligation is thus conceivable also in *B. subtilis* cytochrome b_{558}. However, although it is suggested from the protoheme-to-flavin ratio in isolated SDH complex (11), the sequence does not indicate the presence of two hemes in cytochrome b_{558}; pairs of histidines on two predicted α-helical membrane-spanning protein segments that could coordinate two hemes are not evident, as in the case of the complex III-type cytochrome *b*.

The heme ligands in cytochromes can be determined with the help of various biophysical techniques. These methods require large amounts of protein, and in some cases the cytochrome needs to be specifically labeled with enriched isotope. Both the production of large quantities of protein and the labeling are facilitated if native cytochrome can be expressed at high levels in a microorganism with simple growth requirements. Wild-type and mutant *B. subtilis* cytochromes b_{558} produced in *E. coli* are presently used for more detailed structural studies of cytochrome b_{558}.

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