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Bacillus subtilis Mutant Succinate Dehydrogenase Lacking Covalently Bound Flavin: Identification of the Primary Defect and Studies on the Iron–Sulfur Clusters in Mutated and Wild-Type Enzyme†

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ABSTRACT: Succinate dehydrogenase consists of two protein subunits and contains one FAD and three iron–sulfur clusters. The flavin is covalently bound to a histidine in the larger, Fp, subunit. The reduction oxidation midpoint potentials of the clusters designated S-1, S-2, and S-3 in *Bacillus subtilis* wild-type membrane-bound enzyme were determined as +80, −240, and −25 mV, respectively. Magnetic spin interactions between clusters S-1 and S-2 and between S-1 and S-3 were detected by using EPR spectroscopy. The point mutations of four *B. subtilis* mutants with defective Fp subunits were mapped. The gene of the mutant specifically lacking covalently bound flavin in the enzyme was cloned. The mutation was determined from the DNA sequence as a glycine to aspartate substitution at a conserved site seven residues downstream from the hisitidine that binds the flavin in wild-type enzyme. The redox midpoint potential of the iron–sulfur clusters and the magnetic spin interactions in mutated succinate dehdyrogenases were indistinguishable from the those of the wild type. This shows that flavin has no role in the measured magnetic spin interactions or in the structure and stability of the iron–sulfur clusters. It is concluded from sequence and mutant studies that conserved amino acid residues around the histidyl-FAD are important for FAD binding; however, amino acids located more than 100 residues downstream from the histidyl in the Fp subunit can also effect flavinylation.

Succinate dehydrogenase is a membrane-bound iron–sulfur flavoenzyme found in aerobic cells. It catalyzes the oxidation of succinate to fumarate and is located on the matrix side of the inner membrane of mitochondria and on the inner side of the cytoplasmic membrane in procaryotic cells. The catalytic portion of the enzyme is composed of two different protein subunits (Hederstedt & Rutberg, 1981; Hatefi, 1985; Condon et al., 1985). The larger subunit (M_r 64 000–72 000) is designated as Fp and contains a covalently bound flavin adenine dinucleotide (FAD)[1] in 8α-*N*(3)-histidyl linkage to the protein (Salach et al., 1979). The smaller subunit (M_r 25 000–30 000) is designated as Ip.

The structural genes for the Fp and the Ip subunit of *Escherichia coli* (*sdhA* and *sdhB*) (Wood et al., 1984; Darlinson & Guest, 1984) and *B. subtilis* (*sdhB* and *sdhC*) (Philips, Magnusson, Rutberg and Guest, unpublished observations) have been sequenced. The predicted amino acid sequences of the two bacterial enzymes show extensive homologies, and the sequence around the putative FAD binding histidyl in the Fp protein is almost identical with that in bovine heart succinate dehydrogenase (Kenney et al., 1972).

Studies, principally with mitochondrial succinate dehydrogenase, have shown that there are three iron–sulfur clusters, designated as S-1, S-2, and S-3 in the enzyme (Ohnishi & Salerno, 1982; Beinert & Albracht, 1982; Johnson et al., 1985). Clusters S-1 and S-3 can be reduced with succinate, but cluster S-2 can only be reduced with a strong reductant such as dithionite. Cluster S-1 is a binuclear $[2Fe\text{-}2S]^{2+,1+}$ (Salerno et al., 1977; Albracht & Subramanian, 1977) and S-3 is a trinuclear [3Fe-*X*S] type cluster (*X* is 3 or 4) (Ackrell et al., 1984; Johnson et al., 1985). Recently cluster S-2 has been identified and shown to be a tetranuclear $[4Fe\text{-}4S]^{2+,1+}$ type cluster (Johnson et al., 1985; Maguire et al., 1985). Where the clusters are located in relation to the Fp and Ip subunits has not yet been established (Ohnishi & Salerno, 1982; Beinert & Albracht, 1982; Hederstedt et al., 1985).

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[1] Abbreviations: CAP, chloramphenicol; EPR, electron paramagnetic resonance; FAD, flavin adenine dinucleotide; Fp, flavoprotein subunit of succinate dehydrogenase; Ip, smaller protein subunit of succinate dehydrogenase; MCD, magnetic circular dichroism; PA, purification agar; Tc, tetracycline.

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Each of the four electron carriers in succinate dehydrogenase (three iron–sulfur clusters and the FAD) has at least one paramagnetic state. An understanding of magnetic interactions that occur between the single electron carriers within this enzyme may clarify the mechanisms of electron flow within this complex enzyme system. Magnetic interactions have been demonstrated between the FAD and cluster S-1 (Ohnishi et al., 1981) by showing spin relaxation changes in the flavin semiquinone radical EPR signal upon reduction of S-1. They have been demonstrated between clusters S-1 and S-2, which have a characteristic spin interaction, resulting in a fast relaxation of cluster S-1 upon reduction of cluster S-2 (Ohnishi et al., 1973, 1974, 1976). A spin interaction between clusters S-1 and S-3 has been shown from studies on succinate dehydrogenase from the Gram-positive bacterium *Micrococcus luteus* (Crowe et al., 1983). This interaction could be observed by virtue of the reversed relative midpoint potentials of cluster S-1 and S-3 in *M. luteus*, compared to those in mitochondria. It was of interest to determine if these atypical redox potentials in succinate dehydrogenase are exceptional to *M. luteus* or common to aerobic Gram-positive bacteria as speculated by Crowe et al. (1983).

Succinate dehydrogenase from the Gram-positive bacterium *B. subtilis* has been shown, by using EPR, to contain clusters S-1 and S-3. The presence of cluster S-2 can be inferred from the spin-relaxation changes of cluster S-1 at low poised potentials (Hederstedt et al., 1985; Maguire et al., 1985).

B. subtilis mutants specifically lacking covalently bound flavin in succinate dehydrogenase have been isolated (Hederstedt, 1983). One mutant of this type, KA97124, was previously shown to contain all three iron–sulfur clusters in the defective membrane-bound dehydrogenase (Hederstedt et al., 1985). Clusters S-1 and S-3 in the mutated enzyme can be reduced by reversed electron transfer from the respiratory chain, i.e., by NADH, but they cannot be reduced by succinate. This mutant provides an opportunity to study flavin binding to the Fp peptide and to analyze the influence of the FAD on the iron–sulfur clusters by measuring physical properties of the iron–sulfur clusters in the absence of bound FAD. The base pair change of the point mutation *sdhB124*, which is located in the gene for the Fp subunit and which results in the lack of flavin in the succinate dehydrogenase in strain KA97124, is described. This study also evaluates the redox midpoint potentials of the three iron–sulfur clusters and the magnetic spin interactions between clusters S-1 and S-2 and between clusters S-1 and S-3, in the *B. subtilis* wild-type enzyme, the flavin-deficient enzyme, and an additional mutated enzyme which is enzymatically inactive.

Materials and Methods

Bacillus subtilis Strains and Membrane Preparation. The *B. subtilis* strains 168 (prototrophic), KA98011 (*trpC2 sdhB11*) (Ohnē et al., 1973), KA97101 (*trpC2 leu-2 sdhB101*), KA97124 (*trpC2 leu-2 sdhB124*), and KA97129 (*trpC2 leu-2 sdhB129*) (Hederstedt et al., 1982) were used. The Sdh phenotype was checked on purification agar (PA) plates (Carls & Hanson, 1971). The cultivation of bacteria and preparation of cytoplasmic membranes were done as recently described (Hederstedt et al., 1985). Membrane preparations were tested for succinate dehydrogenase activity at 38 °C with phenazine methosulfate and 2,6-dichlorophenolindophenol as electron acceptors as described (Hatefi, 1978) but in the presence of 2.5 mM KCN. The succinate dehydrogenase activity of wild-type membranes was 3.1 μmol min^{-1} (mg of protein)$^{-1}$, while mutant membranes had activities of less than 0.01 μmol min^{-1} (mg of protein)$^{-1}$. Membranes were also analyzed for their content of succinate dehydrogenase protein by immunoelectrophoresis against Fp-specific rabbit antiserum (Hederstedt & Rutberg, 1980). Protein was determined by the method of Lowry et al. (1951) in the presence of 1.7% (w/v) sodium dodecyl sulfate and with serum albumin as a standard.

Plasmids. Plasmids pKIM31 and pKIM57 are derivatives of pHV32 (*cat tet amp*) (Niaudet et al., 1982) carrying fragments of the *B. subtilis sdh* operon cloned in the *Pst*I and the *Hin*dIII–*Eco*RI sites, respectively (Magnusson et al., 1985) (see Figure 1). These plasmids cannot replicate but express chloramphenicol (CAP) resistance in *B. subtilis*. The plasmids replicate and express CAP (12.5 μg mL^{-1}) and tetracycline (Tc) (15 μg mL^{-1}) resistance in *E. coli*.

DNA Cloning and Sequencing. Bacillus subtilis competent cells were prepared according to Arwert and Venema (1973). Extraction of the chromosomal or plasmid DNA, agarose gel electrophoresis, restriction, and ligation of the DNA were done as described by Maniatis et al. (1982).

For cloning of *sdhB124* plasmid pKIM31 was integrated into the chromosome by homologous recombination next to the *sdhB* gene by transforming strain KA97124 with plasmid DNA. Transformants were selected on Tryptose Blood Agar Base (Difco Laboratories, Detroit, MI) plates containing 5 μg of CAP mL^{-1}. *Eco*RI-digested chromosomal DNA from one transformant was diluted to 10 μg of DNA mL^{-1}, ligated, and used for transformation (Hanahan, 1983) of *E. coli* 5K (*hsdR hsdM thi thr rpsL lacZ*) (obtained from L. O. Heden, KabiGen AB, Stockholm). Plasmid pKIM124 was isolated from one CAP and Tc-resistant *E. coli* clone. The *sdhB* gene fragment was sequenced by the dideoxy chain terminating method after subcloning the *B. subtilis Bam*HI–*Eco*RI DNA fragment (see Figure 1) into phage M13 mp8 and mp9 (Sanger et al., 1977; Messing et al., 1981). Restriction endonucleases and phage T4 DNA ligase were from the New England Biolabs, Beverly, MA. Two specific 15-mer oligonucleotides used as primers for DNA sequencing were synthesized by Hans Hultberg, KabiGen AB, Stockholm.

Redox Titrations and EPR Spectroscopy. The redox titrations were performed in an anaerobic cell at 30 °C, flushed with oxygen-free argon. The measured potential was obtained by using platinum and calomel electrodes, calibrated with quinhydrone at pH 7.0. Membrane preparations were about 20 mg of protein/mL, suspended in 0.1 M potassium phosphate, pH 7.0. Mediator dyes were selected to cover the range of the titration and were present at a concentration of 10 μM. The dyes used were methylene blue, 1,4-naphthoquinone-2-sulfonate, anthroquinone-2,6-disulfonate, 2-hydroxy-1,4-naphthoquinone, quinhydrone, 1,2-naphthylquinone, phenazine methosulfate, duroquinone, anthroquinone-1,5-disulfate; 2-hydroxynaphthoquinone, benzylviologen, and triquat. The samples were reduced and oxidized with sodium dithionite and potassium ferricyanide solutions (0.1 M) which were prepared in anaerobic potassium phosphate (0.1 M) at pH 7.0. Samples were removed with a gas-tight syringe and placed in anaerobic, calibrated EPR tubes and rapidly frozen in a freezing mixture of isopentane and methylcyclohexane (1:1).

Electron paramagnetic resonance measurements were made on a Varian E-109-E spectrometer, and the samples were cooled with liquid helium by using an Air Products Heli-Tran flow system. Spectra were recorded on a DEC PDP-11/34 computer for subsequent base-line correction.

Results

Genetic Mapping of sdhB Mutations. The *sdh* operon in *B. subtilis* contains three genes, *sdhA*, *sdhB*, and *sdhC*, which

Table I: Properties of Four *B. subtilis* Fp (*sdhB*) Mutants[a]

strain	*sdh* mutation	mutation located at the N-terminal part of the Fp polypeptide[b]	covalently bound flavin in Fp polypeptide	iron–sulfur cluster S-1	iron–sulfur cluster S-2	iron–sulfur cluster S-3	location in the cell of Fp and Ip polypeptides[c]
KA98011	*sdhB11*	no	–	–	ND	ND	cytoplasm
KA97101	*sdhB101*	yes	+	+	+	+	membrane
KA97124	*sdhB124*[d]	yes	–	+	+	+	membrane
KA97129	*sdhB129*	no	–	+	+	+	membrane

[a] Data from this work, Hederstedt (1983), and Hederstedt et al. (1985). Determination of clusters S-1, S-2, and S-3 in KA97129 by L. Hederstedt and T. Ohnishi (unpublished observations). ND, not determined; (+) present; (–) absent. [b] The amino-terminal end fragment encompassing amino acid residues 1–158 of the Fp polypeptide. [c] The polypeptides have the same apparant molecular weight (65K and 28K) as in the wild type as determined by sodium dodecyl sulfate gel electrophoresis. [d] The mutation sequenced in this work. See Figure 2.

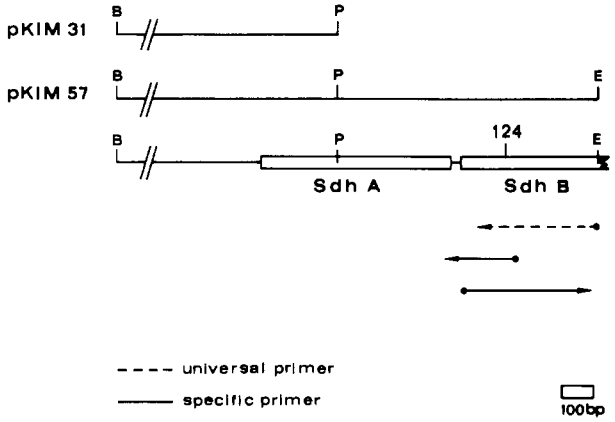

FIGURE 1: Genetic map of the first part of the *sdh* operon in *B. subtilis*, the chromosomal DNA inserts in plasmid pKIM31 and pKIM57 and DNA sequencing. The vector, pHV32, is not shown. Cytochrome b_{558} and the Fp polypeptide of succinate dehydrogenase are coded for by the *sdhA* and the *sdhB* genes, respectively. The location of the mutation sdhB124 is indicated. The directions and the extent of DNA sequencing by the dideoxy method is shown with arrows. B, P, and E designate *Bam*HI, *Pst*I, and *Eco*RI endonuclease restriction sites.

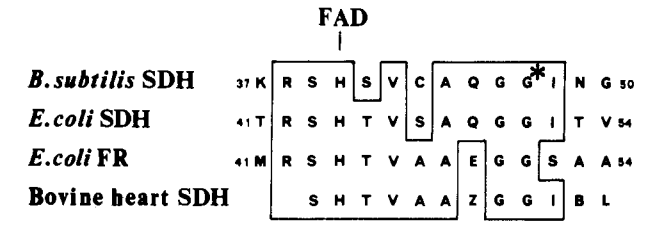

FIGURE 2: Amino acid sequence comparison of the histidyl–flavin region in the Fp subunit of four succinate oxidoreductases. The asterisk indicates the amino acid residue which, when replaced by an aspartate residue due to the mutation *sdhB124*, results in a flavin-deficient membrane bound *B. subtilis* succinate dehydrogenase (SDH). The sequence for the bovine heart protein has been determined from the isolated flavin peptide (Kenney et al., 1972), whereas the *E. coli* (Cole, 1982; Wood et al., 1984) and *B. subtilis* (Philips, Magnusson, Rutberg, and Guest, unpublished results) sequences are predicted from the nucleotide sequence. FR is fumarate reductase.

are transcribed in that order and code for cytochrome b_{558}, Fp, and Ip, respectively, of the succinate dehydrogenase–cytochrome b_{558} complex (Hederstedt et al., 1982; Magnusson et al., 1983). Point mutations *sdhB11*, *sdhB101*, *sdhB124*, and *sdhB129* have been located to the structural gene for the Fp subunit by classical transformation crosses, by complementation analysis and by analysis of the biochemical properties of strains containing these mutations (Hederstedt et al., 1982; Hederstedt, 1983). The four *sdhB* mutations were mapped more precisely by transforming strains containing the respective mutation to CAP resistance with plasmids pKIM57 and pKIM31. The former plasmid carries the *sdhA* gene and 477 base pairs of the adjacent *sdhB* gene. This part of the *sdhB* gene codes for the amino-terminal end of the Fp subunit containing the histidine that is flavinylated. Plasmid pKIM31 only carries a fragment of the *sdhA* gene and was used as a control and for cloning (see Figure 1). The CAP-resistant transformants were tested on PA plates for insertional marker rescue of the *sdhB* mutation by the plasmid. Sdh-positive CAP-resistant cells were obtained with pKIM57 and strains containing mutation *sdhB101* or *sdhB124*. We conclude that both of these mutations must be located on the 477 base pair end fragment of *sdhB*. No marker rescue of *sdhB11* or *sdhB129* was obtained. Thus, these mutations are most likely situated downstream from the first *Eco*RI restriction site in the *sdhB* gene (Figure 1).

DNA Sequencing of sdhB124. Mutation *sdhB124* was cloned in plasmid pKIM124 as described under Materials and Methods. *E. coli* 5K containing this plasmid formed reddish colonies due to the production of cytochrome b_{558} from the co-cloned *B. subtilis sdhA* gene (Magnusson et al., 1985). The cloning of the correct DNA fragment was verified by restriction enzyme mapping. Transformation of the wild-type *B. subtilis* with pKIM124 resulted in both Sdh^+ and Sdh^- CAP-resistant cells. Furthermore, strain KA97124 (*sdhB124*) could not be transformed to Sdh^+ with pKIM124. These results are as expected if pKIM124 carries the *sdhB124* mutation. The *sdhB* DNA fragment was sequenced as outlined in Figure 1. The mutation was found to be a G to A transition which is predicted to result in a replacement of a conserved glycine by an aspartate at amino acid position 47 in the Fp polypeptide (Figure 2). This amino acid substitution results in a membrane-bound succinate dehydrogenase lacking covalently bound flavin.

Redox Titration of Iron–Sulfur Clusters in Wild-Type and Mutated Succinate Dehydrogenase. To determine the effects of the flavin deficiency on the redox midpoint potentials and the magnetic interactions of the iron–sulfur clusters in the dehydrogenase, both wild-type and mutated enzymes were studied by using EPR spectroscopy. The relevant properties of the mutants analyzed are summarized in Table I.

An EPR spectrum of dithionite-reduced *B. subtilis* wild-type membranes is shown in Figure 3A. The signals at g values of 2.035, 1.94, and 1.89 are also obtained from membranes reduced with succinate and are from iron–sulfur cluster S-1 in succinate dehydrogenase (Hederstedt et al., 1985). The EPR spectrum of cluster S-3 is detected in oxidized *B. subtilis* membranes at temperatures below 15 K. This spectrum, which has a derivative peak centered around g 2.01, was recently published [Figure 6 of Hederstedt et al. (1985)] and is not shown here. The $g = 2.0$ centered derivative peak seen in Figure 3A is from free radical(s) and is not cluster S-3. Membrane components, other than succinate dehydrogenase, do not contribute significantly to the EPR signals in reduced and oxidized *B. subtilis* membranes, as shown by the lack of

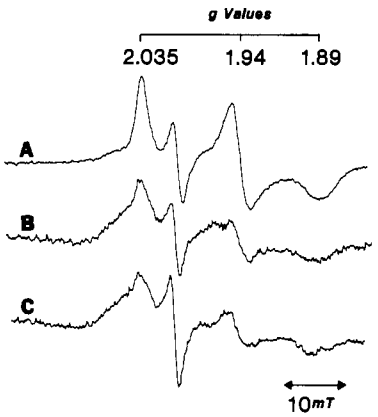

FIGURE 3: EPR spectra of dithionite-reduced membranes of wild-type bacteria (A), succinate dehydrogenase mutants KA97124 (B), and KA97101 (C). The spectra were recorded at 20 K, at 20-mW microwave power, and with 1-mT modulation amplitude at 100 KHz, and the scan rate was 1.25 mT/min. The spectra have been corrected for base-line variation and normalized for slight differences in protein concentration and EPR tube diameter. The relative gain of spectrum A to B and C is 0.5. the g values indicated correspond to those of cluster S-1 in *B. subtilis* succinate dehydrogenase (Hederstedt et al., 1985).

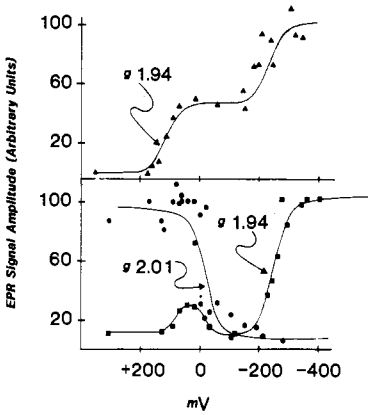

FIGURE 4: Redox titration of iron–sulfur clusters from succinate dehydrogenase in wild-type and *B. subtilis* membranes. EPR spectra were recorded essentially as in Figure 1. The upper panel shows a titration of the g = 1.94 signal of cluster S-1 (▲) measured at 10 mW and 20 K. The lower panel shows titrations of the g = 1.94 signal of cluster S-1 (■) and the g = 2.01 signal of cluster S-3 (●), both measured at 8 K and 20 mW of power. The individual data points have been fitted to a Nernst n = 1 equation ideal curve.

these signals in membranes from mutants that totally lack succinate dehydrogenase (Hederstedt et al., 1985).

Dithionite-reduced membranes of KA97124 and of KA97101 (the mutant with a catalytically inactive, flavin-containing, succinate dehydrogenase) showed EPR signals with the same g values as those of the wild type (Figure 3B,C). Membranes of the two mutants contain lower amounts of succinate dehydrogenase protein than wild type and elicit correspondingly weaker EPR signals as compared to the wild-type membranes (Hederstedt, 1983; Hederstedt et al., 1985).

In a redox titration of membranes from the wild-type strain plotting the signal height of the g = 1.94 derivative peak, recorded at 20 K and 10-mW microwave power, vs. the potential, two components with midpoint potentials of +80 and −240 mV are noted (Figure 4). Approximately the same midpoint potentials were obtained if the signals at g = 2.035 or g = 1.89 were measured. The E_m = +80 mV component is cluster S-1 whereas the E_m = −240 mV component represents an increased spin-relaxation rate of cluster S-1 which

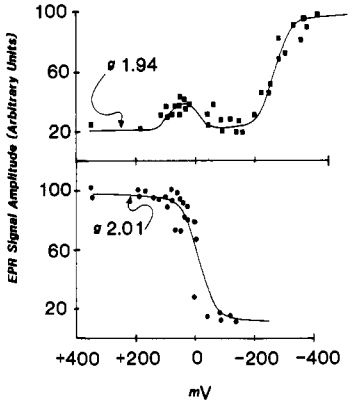

FIGURE 5: Redox titrations of membranes of *B. subtilis* mutant KA97124, which lacks covalently bound flavin in the membrane-bound succinate dehydrogenase. The g = 1.94 signals [upper panel (■)] and the g = 2.01 signals [lower panel (●)] were recorded at 8 K and 20 mW. Other EPR conditions were the same as in Figure 1. The individual data points have been fitted to a Nernst n = 1 equation ideal curve.

results when cluster S-2 becomes reduced (Ohnishi et al., 1976; Maguire et al., 1985). This measurement provides the midpoint potential of cluster S-2. The EPR signal of cluster S-3 was recorded at 8 K, measuring the derivative peak centered at g = 2.01, and has a midpoint potential of −25 mV (Figure 4). The amplitude of the g = 1.94 signal in EPR spectra recorded at 8 K and 20-mW microwave power showed a transient decrease in the potential interval from 0 to −200 mV, when the spectra were recorded at saturating power for cluster S-1. This decrease was not observed in spectra recorded at 20 K, and the decrease at around 0 mV coincides with the reduction of cluster S-3. This same phenomenon was previously observed in *M. luteus* succinate dehydrogenase and was interpreted as a spin interaction between clusters S-1 and S-3 (Crowe et al., 1983). The reason for the decrease in the height of the g = 1.94 signal in Figure 4 at poised potentials below 25 mV is due to cluster S-1 having a slower spin relaxation when cluster S-3 is reduced. Spectra recorded under nonsaturating conditions (10-μW microwave power) measuring cluster S-1 show no decrease in signal height following reduction of S-3 (spectra not shown). Attempts were made to see if there was any change in the spin relaxation of cluster S-3 when cluster S-1 is oxidized and reduced. Cluster S-3 has a rapid spin relaxation, and it was not possible in the membrane preparations used in this study to resolve any such changes upon reduction of cluster S-1.

A redox titration of the membranes of mutant KA98011 (which specifically lacks membrane-bound succinate dehydrogenase), recorded at 10 and 20 K, measuring the EPR signal heights centered around g = 1.94 and 2.01 showed no signal change at potentials of +80, −25, and −240 mV (spectra not shown).

Figure 5 shows the redox titration of membranes from mutant KA97124 measuring the signal heights of the g = 1.94 (S-1 and S-2) and the g = 2.01 (S-3) features. The redox midpoint potential of cluster S-1 was measured at 20 K (not shown) where the EPR signal of this cluster is well resolved. The three iron–sulfur clusters in the mutant were found to have the same midpoint potentials as those of the wild type, and the spin interactions between clusters S-1 and S-2 were not altered in agreement with previous power saturation measurements [Table II of Hederstedt et al. (1985)]. The same results were obtained with membranes from mutant KA97101. Additionally, a spin interaction between cluster S-1 and S-3 was indicated in both mutants; i.e., the g = 1.94 signal am-

plitude, measured at 8 K and at power-saturating conditions, showed a decrease in the potential interval from 0 to −200 mV.

Discussion

The redox midpoint potentials of the iron–sulfur clusters in *B. subtilis* membranes, measured in this study, can be described unequivocally as being from succinate dehydrogenase, as these same clusters are specifically missing in membranes of mutants which lack membrane-bound succinate dehydrogenase. The apparent midpoint potentials of clusters S-1 (E_m = +80 mV), S-2 (E_m = −240 mV), and S-3 (E_m = −25 mV) are similar to those of succinate dehydrogenase in *M. luteus* (Crowe et al., 1983), but they are different from the +50 to 0, −150 to −260, and +60 to +80 mV midpoint potentials of the membrane-bound enzyme in bovine heart mitochondria (Ohnishi & Salerno, 1982) and Gram-negative bacteria (Condon et al., 1985; Ingledew & Prince, 1977; Zannoni & Ingledew, 1983). The main difference is that cluster S-3 has a lower midpoint potential than S-1 in the enzymes of the two Gram-positive bacteria. The reason for this can only speculated upon. Possibly, as suggested by Crowe et al. (1983), it is because Gram-positive bacteria contain menaquinone in their membranes, whereas Gram-negatives contain both ubiquinone and menaquinone. Menaquinone has a lower redox midpoint potential than ubiquinone, and a lower potential for cluster S-3 (which is thought to donate electrons to quinone) would facilitate electron transfer in the bacterial enzyme.

Interactions between cluster S-3 and ubiquinone have been detected in mitochondrial membranes (Ruzika et al., 1975; Ingledew et al., 1976). It has not yet been determined whether a similar interaction also occurs in membranes of Gram-positive bacteria.

The detectable magnetic spin interaction between cluster S-1 and S-3 occurs between the reduced form of S-1 and the oxidized form of S-3. Cluster S-1 has an enhanced spin relaxation at poised potentials where S-3 is oxidized, and this was detected in these experiments as an increased EPR signal height when measuring under power saturating conditions. The spin relaxation of cluster S-1 decreases as cluster S-3 becomes reduced. Cluster S-3 is a trinuclear cluster which has a paramagnetic state when it is both oxidized and reduced (Johnson et al., 1985; Ackrell et al., 1984). Cluster S-3 in the oxidized state has a Kramer's $S = {}^1/_2$ spin system, which is similar to many other reduced ferredoxins.

Cluster S-3 being paramagnetic in both the oxidized and the reduced form is similar to other trinuclear iron–sulfur clusters that have been studied. In the reduced form these trinuclear clusters have a non-Kramer's $S = 2$ spin system, which is strongly paramagnetic (Huynh et al., 1980; Thomson et al., 1981). No obvious magnetic spin interaction occurs between reduced cluster S-3 and reduced cluster S-1, even though reduced S-3 is more strongly paramagnetic than its oxidized form. Preliminary findings on the beef enzyme cluster S-2 (Maguire et al., 1985) suggest that the reduced form of this cluster likely has an $S = {}^1/_2$ spin system and not a mixed spin state as has recently been demonstrated in another tetranuclear Fe-S cluster from *Azotobacter vinlandii* nitrogenase (Lindahl et al., 1985). It is of interest to note that in succinate dehydrogenase two distinct clusters with an $S = {}^1/_2$ spin system (oxidized S-3 and reduced S-2) interact with reduced cluster S-1 and cause an enhanced spin relaxation. Certainly more studies are needed to understand magnetic interactions within this enzyme as it relates to the relative positioning of the clusters and to the mechanisms of electron exchange between clusters.

The succinate dehydrogenase of *B. subtilis* mutants KA97124 and KA97101 with defective Fp subunits contained clusters S-1, S-2, and S-3 with normal redox midpoint potentials, showed normal S-1 and S-3 EPR spectra, and had the same measurable spin interactions as in the native enzyme. These findings were unexpected for the flavin-deficient enzyme, considering the influence the relatively large FAD molecule might have on the structure of the Fp subunit and the spin interactions that have been demonstrated between the flavin semiquinone radical and cluster S-1 in the mammalian enzyme (Ohnishi et al., 1981).

The primary defect in the flavin-deficient succinate dehydrogenase of strain KA97124 (*sdhB124*) was, from the DNA sequence, determined as a glycine to aspartate substitution in the Fp polypeptide at position 47, seven residues downstream from the putative histidyl to which flavin is covalently bound in the wild-type subunit. The two glycines at positions 46 and 47 in the *B. subtilis* Fp subunits are conserved in other succinate dehydrogenases and in fumarate reductase (Figure 2). They also seem conserved in *p*-cresol methylhydroxylase, which contains 8α-*O*-tyrosyl bound FAD (Singer & McIntire, 1984), but are not apparent in trimethylamine dehydrogenase containing 6-*S*-cysteinyl-bound FMN. (Steenkamp et al., 1978; Kenney et al., 1978) and in different enzymes with noncovalently bound FAD [Table III of Wood et al. (1984)]. Since the primary sequence around the flavin attachment site is known for just a few functionally different enzymes, one can only speculate that the two glycines are essential for maintenance of a peptide structure capable of flavinylation. The mutated protein seems defective not only in covalent but also in noncovalent binding of the FAD. This could be due to a steric interference from the aspartate and/or electrostatic alteration of the flavin binding site by the aspartate. This is supported by two observations: (1) The succinate dehydrogenase protein in KA97124 lacks both covalently and noncovalently bound flavin as determined by electrophoresis under conditions where the dinucleotide is not dissociated from other enzymes containing noncovalently bound FAD (Hederstedt, 1983). (2) Succinate dehydrogenase activity was not obtained when membranes from KA97124 were preincubated with FAD (2.7 mM; 11 mg of membrane protein mL^{-1}) before assay or when FAD (0.55 mM) was included in the cuvette buffer. The same treatments did not effect the activity in wild-type membranes.

It is not known by what mechanism flavin is covalently bound to protein at specific sites (Singer & Mcintire, 1984; Decker, 1982). Host-specific factors do not seem required (Brandsch & Bichler, 1985). The Fp polypeptide is the only protein in *B. subtilis* modified by covalent flavinylation (Hederstedt, 1983). The subunit is modified independently of the Ip subunit and before the subunits are assembled into a membrane-bound succinate dehydrogenase (Hederstedt & Rutberg, 1980).

Proteins with different functions but containing the 8α-*N*-(3)-histidyl-FAD do not exhibit amino acid sequence homologies around the flavin binding histidine (Singer & McIntire, 1984; Shiga et al., 1983; Cook et al., 1985). However, succinate oxidoreductases containing these types of modifications show almost identical sequences in the flavin–histidyl region (Figure 2). The domain in the Fp subunit of these enzymes that binds the ADP moiety of FAD is probably formed by the amino-terminal end of the protein, upstream from the flavin binding hisitidine (Wood et al., 1984). Interestingly point mutations outside and far downstream from the histidyl–flavin region can prevent flavin from being covalently bound. This

is demonstrated by the properties of mutants KA97129 (*sdhB129*) and KA98011 (*sdhB11*) (Table I). These mutants most likely contain single, rather than double, point mutations in the *sdhB*. This is supported because both mutants revert to an Sdh-positive phenotype at about the same frequency as strain KA97124. Furthermore, mutation *sdhB11* can be separated in reciprocal three factor transformation crosses and mapped relative to other *sdhB* mutations located both upstream, e.g., *sdhB101*, and downstream of *sdhB11* (Ohnē et al., 1973; Hederstedt et al., 1982). The Fp polypeptide in both mutants lacks covalently bound flavin and is mutated at a point distant from the flavin binding histidine. In KA98011 the mutation also causes lack of iron–sulfur cluster S-1 and defective membrane binding of Fp and Ip presumably because of an altered protein structure. We conclude that the amino-terminal approximately 150 amino acid residue of the Fp polypeptide including the flavinylation site region is not sufficient for flavin to be bound in *B. subtilis*.

The data from the mutants suggest that amino acid residues from several different parts of the Fp polypeptide contribute to the "binding pocket" of the flavin mononucleotide moiety of FAD, which is similar to results from glutathione reductase (Schultz et al., 1982). Extensive protein folding of the larger subunit of *B. subtilis* succinate dehydrogenase to form this flavin binding site is most likely required before the cofactor can be covalently attached to the appropriate histidine.

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Photosynthetic Electron Transfer in the Absence of Cytochrome c_2 in *Rhodopseudomonas capsulata*: Cytochrome c_2 Is Not Essential for Electron Flow from the Cytochrome bc_1 Complex to the Photochemical Reaction Center

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ABSTRACT: Photosynthetic electron flow from the cytochrome bc_1 complex to the reaction center has been studied in a strain of *Rhodopseudomonas capsulata* which has had the gene for cytochrome c_2 deleted from its genome. Previously, cytochrome c_2 was thought to be essential for electron flow between these two complexes, but we find this not to be the case in *R. capsulata*. Indeed, in this organism it seems likely that cytochrome c_1 is able rapidly ($t_{1/2} < 100$ μs) to transfer electrons directly to the reaction center. However, this reaction is incomplete; only some 20% of the reaction centers are reduced in this way. In the wild type, a further 20% is rapidly reduced by cytochrome c_2, but the remaining reaction centers are reduced rather more slowly by an as yet unidentified route that may involve cytochrome c_2 shuttling between complexes. The deletion of the cytochrome c_2 gene allows a determination of the oxidation–reduction midpoint potential of cytochrome c_1 in the absence of cytochrome c_2: cytochrome c_1 has an E_{m7} of 345 mV. Furthermore, the finding that a phase of the electrogenic carotenoid bandshift accompanies the oxidation of cytochrome c_1 in the absence of c_2 indicates that the heme of cytochrome c_1 must be near the inner aqueous–membrane interface of the chromatophore.

Photosynthetic electron transfer in *Rhodopseudomonas capsulata* involves two membrane-associated complexes: the photochemical reaction center and the cytochrome bc_1 complex [for recent reviews, see Prince et al. (1982) and Crofts (1985)]. Both contain several distinct redox centers and together are responsible for electrogenic proton translocation. Up until now, there has been little evidence to suggest that they interact directly with each other; rather, it has been assumed that mobile electron carriers "shuttle" between the two. Ubiquinone is thought to fulfill this role in transferring electrons from the reducing end of the reaction center to the cytochrome bc_1 complex, while cytochrome c_2 is thought to transfer electrons from the bc_1 complex to the reaction center's oxidizing site. Furthermore, cytochrome c_2 is also involved in aerobic growth, where it is thought to function in an analogous fashion between the cytochrome bc_1 complex and the terminal oxidase known as cytochrome b_{410} (Baccarini-Melandri et al., 1978).

Using site-directed mutagenesis, Daldal et al. (1986) have deleted the gene coding for cytochrome c_2 from *R. capsulata* and found that the resulting strain is still capable of photosynthetic and aerobic growth. This paper presents an investigation of the effects of the absence of cytochrome c_2 on photosynthetic electron transfer.

Materials and Methods

Rhodopseudomonas capsulata [*Rhodobacter capsulatus* in the proposed taxonomy of Imhoff et al. (1984)] strain MT-G4/S4, which lacks cytochrome c_2, and the parent strain MT1131, which possesses it, were grown aerobically in shake flasks from frozen stocks until they reached early logarithmic phase in 300 mL, and were then transferred to 1.5-L sealed bottles and grown photosynthetically to late log phase as described previously (Daldal et al., 1986). Chromatophores were prepared by using a French pressure cell following standard procedures and were "purified" on a discontinuous sucrose gradient (10–40% sucrose in 10 mM *N*-morpholinopropanesulfonate, pH 7); spheroplasts were prepared by using lysozyme and ethylenediaminetetraacetate (Prince et al., 1975; Takemoto & Bachmann, 1979).

Flash-induced optical absorbance changes were measured with a rapidly responding double-beam spectrometer constructed by the Bio-Instrumentation Group at the University of Pennsylvania, interfaced via a Nicolet 3091 oscilloscope to an IBM personal computer. Actinic flashes were provided by a xenon flash lamp (full width at half-height about 12 μs)

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