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EPR characterization of soluble fragments of succinate dehydrogenase from mutant strains of *Bacillus subtilis*

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Succinate dehydrogenase is a membrane-bound metallo-flavo-enzyme containing a bi- (S-1), a tri- (S-3) and a tetranuclear (S-2) iron–sulfur cluster. The catalytic portion of the enzyme contains two distinct subunits designated Fp and Ip. Using concentrated extracts from mutant strains of *Bacillus subtilis* it was demonstrated, by using low temperature EPR, that cluster S-2 can be assembled in a soluble succinate dehydrogenase. In a mutant with a truncated Ip subunit which lacks 7 of the 11 conserved cysteine residues, cluster S-1 lacked the spin relaxation properties attributable to an adjacent cluster S-2. These data are consistent with a model where one or more cysteine residues from the middle set of 4 conserved cysteines in the Ip subunit are ligands to the tetranuclear cluster.

Succinate dehydrogenase; Iron–sulfur cluster; Flavo-enzyme; Metallo-enzyme assembly

1. INTRODUCTION

Succinate dehydrogenase (EC 1.3.99.1) is a membrane-bound enzyme that catalyzes the oxidation of succinate to fumarate. It is located on the matrix side of the inner membrane of mitochondria and on the cytoplasmic side of the membrane in aerobic bacteria [1–3]. The catalytic part of the enzyme is composed of two polypeptides, known as the flavin-containing polypeptide (Fp), and a smaller polypeptide known as the iron-containing polypeptide (Ip). There are three iron–sulfur clusters in the enzyme and they are designated as cluster S-1 (a $[2Fe\text{-}2S]^{1+,2+}$ type cluster), S-2 (a $[4Fe\text{-}4S]^{2+,1+}$ type cluster) and S-3 (a [3Fe-4S] type cluster) [4–6].

The sequence of the genes for *Escherichia coli* succinate dehydrogenase and fumarate reductase and *Bacillus subtilis* succinate dehydrogenase are known [7–9]. The partial cDNA sequence for the Ip subunits from several species [10] and the primary structure of the bovine Ip subunit from isolated protein [11] have been determined. *E. coli* fumarate reductase is an enzyme that is very similar to succinate dehydrogenase but catalyzes the reduction of fumarate to succinate during anaerobic growth. Comparison of the Ip amino acid sequences of these enzymes has revealed three sets of conserved cysteine residues which are likely ligands to the iron–sulfur clusters [1,12].

The assembly and detailed structure of succinate dehydrogenase, e.g. which amino acid residues are the ligands to the respective iron–sulfur clusters, are not well understood. The components of the enzyme that are essential for assembly of the prosthetic groups have been studied using *Bacillus subtilis* mutants [13]. These studies have revealed that (i) the Fp and Ip subunits are first synthesized as soluble polypeptides, (ii) iron–sulfur cluster S-1 and covalently bound FAD can be assembled onto the enzyme in the absence of the membrane anchor polypeptide (cytochrome *b*-558), (iii) a full length Ip subunit is not essential for maintenance of cluster S-1 and (iv) FAD is not required for assembly of clusters S-1, S-2 and S-3, with 'native'

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properties [14–16]. It has been recently shown that an *E. coli* fumarate reductase mutant lacking the A subunit (Fp) still contains the binuclear iron–sulfur cluster, demonstrating that this cluster is entirely located on the B (or Ip) subunit [17]. This observation, combined with our findings [14,18] that *B. subtilis* with a truncated Ip containing only the N-terminal set of 4 conserved cysteine residues, has a cluster S-1, strongly suggests that these cysteine residues are the ligands for the binuclear cluster (fig.1). Sequence comparisons to plant ferredoxins of known structure support this conclusion [1,12]. An *E. coli* fumarate reductase mutant with the B (Ip) subunit, truncated at the C-terminus to lack only the third set of conserved cysteines residues, contains the tetranuclear cluster S-2, but only if the A (Fp) polypeptide is present [17].

To study if cluster S-2 is assembled onto soluble *B. subtilis* succinate dehydrogenase subunits and to be able to compare the properties of cluster S-1 in soluble wild type and truncated Ip subunit (fig.1), we have analyzed three succinate dehydrogenase-defective mutants by EPR spectroscopy. In our previous studies [14] on cluster S-1 in soluble enzyme, we utilized whole cell lysates which have the disadvantage of having unidentified broad background EPR signals, and the succinate dehydrogenase cannot be concentrated beyond that found in packed cells. Preparation of concentrated soluble (cytoplasmic) cell extracts made possible more detailed EPR measurements on cluster S-1 and allowed for indirect detection of cluster S-2.

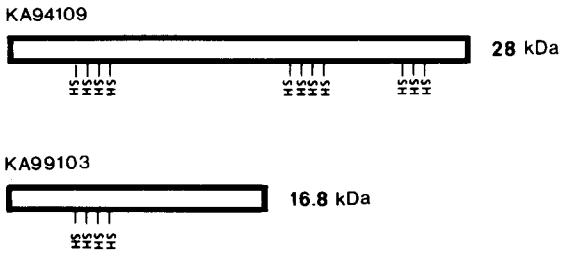

Fig.1. Schematic drawing of the primary structure of cytoplasmic Ip subunit of succinate dehydrogenase from two *B. subtilis* mutants. Mutant KA94109 contains a normal Ip (253 residues) whereas KA99103 contains an Ip which is truncated at the C-terminus as the result of a Trp-147 to stop mutation. The position of the conserved cysteine residues are indicated.

2. MATERIALS AND METHODS

2.1. *Bacterial strains*

The following *B. subtilis* succinate dehydrogenase-defective mutants were used. KA94109 (*sdhC109 ilvB2*) lacks cytochrome *b*-558 and as a result contains wild-type Fp and Ip in the cytoplasm [19]. KA99103 (*sdhB103 leu-2*) contains wild-type Fp and truncated Ip in the cytoplasm [18]. KA97115 (*sdh-115 trpC2 leu-2*) has a mutation in the *sdh* promoter and essentially lacks succinate dehydrogenase protein [19,20]. The succinate dehydrogenase negative phenotype was checked on purification agar plates [21] and by enzyme activity measurements on cell lysates [14].

2.2. *Preparation of concentrated soluble cell extracts*

Cultivation of bacteria and preparation of protoplasts were done as described before [14]. The protoplasts from 1.5 l of culture were lysed by sonication under argon in 1.5 ml of 4°C 0.2 M K-phosphate, 10 mM K-succinate, 10 mM Na-EDTA, 10 mM dithiothreitol (DTT) at pH 7.2. All subsequent steps were performed under argon on ice. The lysate was centrifuged for 30 min at 48000 × *g*. The supernate was transferred to 1 cm diameter dialysis tubing and concentrated by placing the dialysis bag in dry Sephadex G-25. Over a period of 2–4 h the supernate was concentrated about 2-fold. This concentrated soluble extract was analyzed for protein by the Lowry procedure [22] and for succinate dehydrogenase protein by immunoelectrophoresis using anti-succinate dehydrogenase antibodies [13] and purified *B. subtilis* enzyme as the standard.

2.3. *EPR sample preparation and analysis*

The concentrated soluble preparations were transferred anaerobically to calibrated EPR tubes and at room temperature the appropriate substrates were added, prior to freezing the samples in liquid N_2. EPR spectroscopy at X-band was performed on a Varian E-109 spectrometer as described [16]. EPR analysis was done on at least two sets of independently prepared bacterial extracts.

3. RESULTS AND DISCUSSION

Cluster S-1 in membrane bound *B. subtilis* succinate dehydrogenase has a complex spin relaxation process that is altered by at least two adjacent $S = 1/2$ spin systems (reduced cluster S-2 and oxidized cluster S-3) [16]. When succinate is added to the native enzyme, cluster S-3 ($E_{m,7.0} = -25$ mV) and cluster S-1 ($E_{m,7.0} = +80$ mV) are reduced. While cluster S-2 ($E_{m,7.0} = -240$ mV) is not reduced by succinate it can be reduced by dithionite. The EPR signal of cluster S-1 in succinate-reduced enzyme is readily saturated [14]. If cluster S-2 is reduced, cluster S-1 spin relaxation rate is increased and an enhanced EPR signal of cluster S-1 at high power (e.g. 20 mW at 18 K), is detected as compared to a succinate-reduced enzyme. At low, non-saturing power (e.g. 20 μW at 18 K) there is

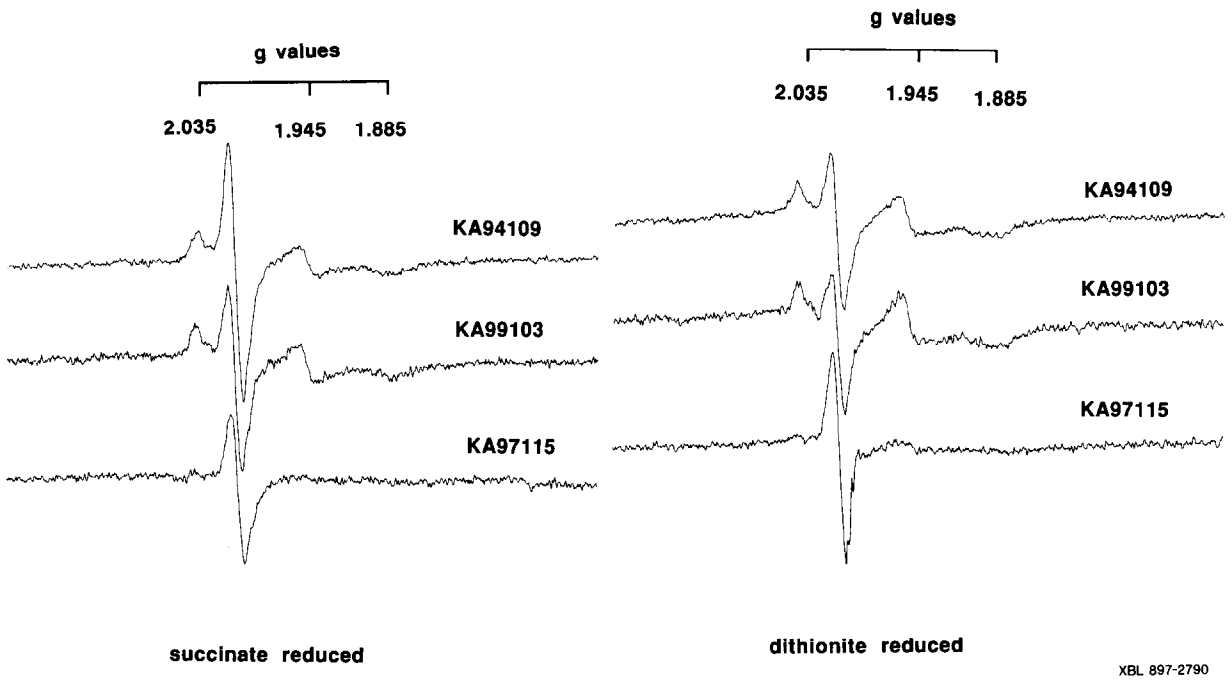

Fig.2. EPR spectra at low microwave power of succinate- and dithionite-reduced soluble extracts of *B. subtilis* succinate dehydrogenase mutants. The EPR settings were: microwave power, 20 μW; temperature, 18 K; modulation amplitude, 1 mT at 100 kHz; scan rate, 12.5 mT/min. Protein concentrations ranged from 62 to 94 mg/ml and succinate dehydrogenase protein in KA94109 and KA99103 was 6–9 μM. Differences in EPR tube diameter and protein concentration were normalized by computation prior to plotting of these spectra. The indicated g values are those of succinate dehydrogenase [14].

no difference in the EPR signal of succinate vs dithionite reduced sample [14,16,23]. The presence of cluster S-2 can thus be inferred from the spin relaxation properties of cluster S-1 in succinate- or dithionite-reduced enzyme. Direct EPR measurement of cluster S-2 is difficult because the signal is very broad and large amounts of protein are required [6,24,25].

EPR spectra of concentrated soluble cell extracts from three *B. subtilis* mutants are shown in figs 2 and 3. At low power, whether as prepared ('succinate reduced') or dithionite-reduced, the samples from mutant strains KA94109 and KA99103 had EPR signals which correspond to that of cluster S-1 (fig.2) [14]. The sample of mutant KA97115 which lacks succinate dehydrogenase protein showed no EPR signal corresponding to that of cluster S-1. The EPR signal centered around g = 2.01 in these spectra is not identified but is *not* related to the succinate dehydrogenase, as it is also present in extracts lacking succinate dehydrogenase protein. Fig.3 shows these same samples measured at a high microwave power where cluster S-1 is saturated if the adjacent cluster S-2 is either not reduced or missing. The EPR spectrum of the KA94109 extract reduced with dithionite and measured at high power was larger than that of the 'succinate-reduced' extract. This is the same as observed with the native membrane-bound enzyme and shows that clusters S-1 and S-2 are present [14,16]. In contrast the EPR spectrum of dithionite-reduced KA99103 extract at high power did not show an increased signal even though at low power it was indistinguishable from that of mutant KA94109 (fig.2). The extracts used in these studies were made in the presence of excess DTT and it is possible that DTT is the reductant of cluster S-1 and not the added succinate. The 'succinate-reduced' KA99103 extract did not have detectable spectral features of cluster S-1 at high power. It is not clear why 'succinate-reduced' KA99103 extracts showed an S-1 EPR spectrum at low power but not at high power as in the KA94109 extracts. However, cluster S-1 was detectable in the

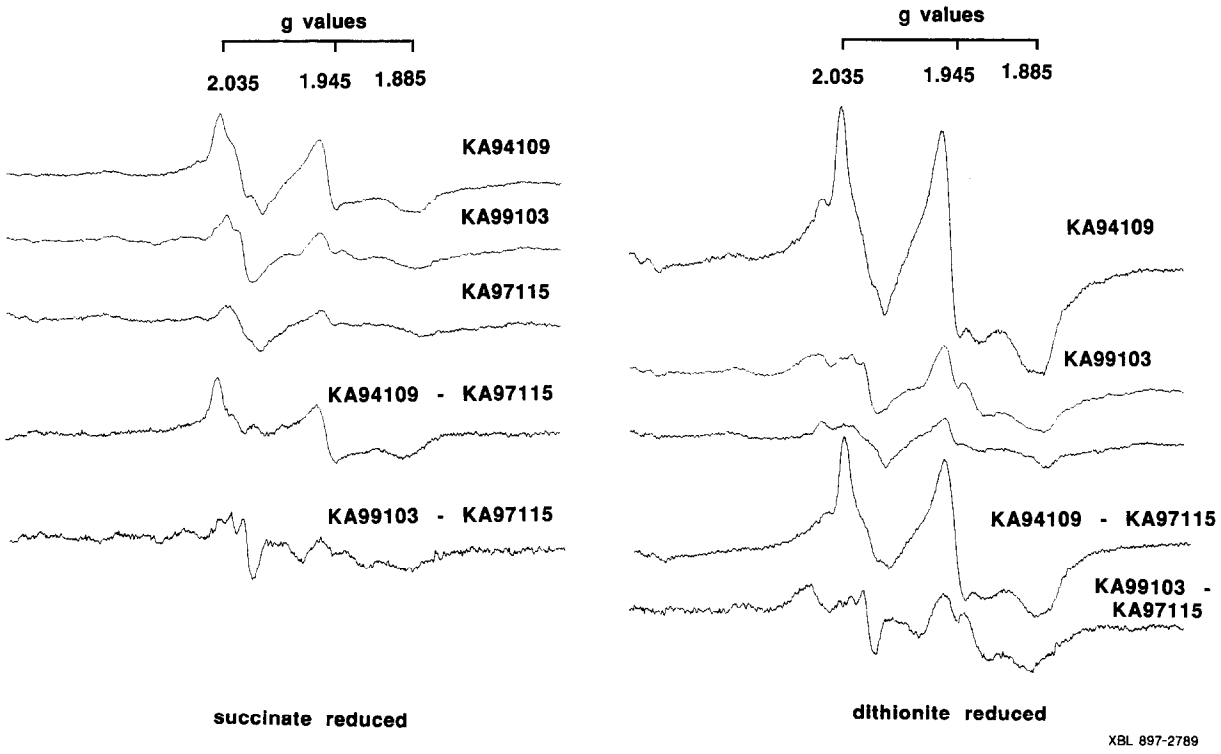

Fig.3. EPR spectra at high microwave power of the same sample as in fig.2. EPR parameters were the same as in fig.2 except that the microwave power was 20 mW and the gain was 0.5 × that of fig.2. Both the original normalized spectra and difference spectra (spectra of extracts of mutant KA97115 subtracted from KA94109 and KA99103) are shown. The indicated g values are those of succinate dehydrogenase [14].

dithionite-reduced KA99103 and it is possible that alterations in the background signal in succinate- vs dithionite-reduced sample mask the detection of cluster S-1 in 'succinate-reduced' KA99103 at 20 mW microwave power, which substantially saturates the signal.

Power saturation measurements of the $g = 1.945$ feature of the S-1 signal of KA94109 extracts reduced with dithionite or succinate and KA99103 extracts reduced with dithionite show that the 'succinate reduced' sample of KA94109 had a much lower spin relaxation rate than the dithionite reduced sample (fig.4). This is consistent with cluster S-2 being present in the KA94109 soluble fraction. Mutant strain KA99103 when reduced with dithionite had a spin relaxation profile very similar to that of 'succinate-reduced' KA94109 extracts indicating that cluster S-2 is not present. The succinate dehydrogenase in these samples con-

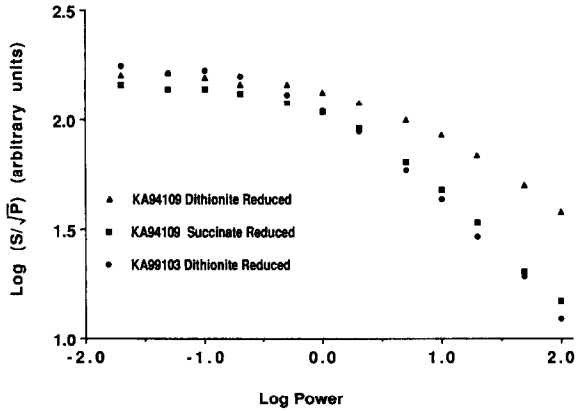

Fig.4. Microwave power saturation profiles of the S-1 EPR signal of soluble succinate dehydrogenase in two *B. subtilis* mutants. The $g = 1.945$ signal height (S) was measured from 20 μW to 100 mW at 18 K. Scan rate was decreased for the low power measurements to 6 mT/min. All other EPR settings were as described in fig.2.

stitutes about 1% of the total protein. 'Contaminating' broad EPR features likely distort the power saturation curves resulting in a power saturation profile of a mixture of signals. However, the relaxation differences between succinate- vs dithionite-reduced KA94109 provides a distinct qualitative measurement for detection of cluster S-2.

We conclude that cluster S-2 like cluster S-1 [14] and the corresponding clusters in fumarate reductase [23], assemble (or at least can be assembled) in soluble subunits before they are bound to the anchor protein in the membrane to form functional enzyme complex. Cluster S-2 is apparently not formed or is not stable in *B. subtilis* containing wild-type Fp and a truncated (16.8 kDa) Ip. This is as expected if the middle conserved set of 4 cysteines provides ligands for cluster S-2 (fig.1).

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