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Ligands to the 2Fe iron-sulfur center in succinate dehydrogenase

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Membrane-bound succinate oxidoreductases are flavoenzymes containing one each of a 2Fe, a 3Fe and a 4Fe iron-sulfur center. Amino acid sequence homologies indicate that all three centers are located in the Ip (B) subunit. From polypeptide and gene analysis of *Bacillus subtillis* succinate dehydrogenase-defective mutants combined with earlier EPR spectroscopic data, we show that four conserved cysteine residues in the first half of Ip are the ligands to the [2Fe-2S] center. These four residues have previously been predicted to be the ligands. Our results also suggest that the N-terminal part of *B. subtilis* Ip constitutes a domain which can incorporate separately the 2Fe center and interact with Fp, the flavin-containing subunit of the dehydrogenase.

Structural mutant; Iron-sulfur protein; *sdh* gene; Succinate oxidoreductase; Nonsense mutation; (*B. subtilis*)

1. INTRODUCTION

Membrane-bound succinate dehydrogenase (SDH) and fumarate reductase (FRD) are iron-sulfur flavoenzymes (EC 1.3.99.1) which catalyse succinate-fumarate interconversion. Their chemical composition and primary structure are conserved (cf. [1–5]). The catalytic part is a dimer with a 60–79 kDa and a 25–31 kDa protein subunit. One FAD is covalently bound to a histidine in the larger flavoprotein (Fp or A) subunit, whereas three iron-sulfur centers seem to be located in the smaller iron-protein (Ip or B) subunit. Studies mainly with mammalian SDH and *Escherichia coli* FRD have demonstrated the centers to be one [2Fe-2S], one [3Fe-*x*S] (where *x* is 3 or 4) and one [4Fe-4S] [1,6–9].

The role of each prosthetic group in electron transfer between the dicarboxylate at the active site on Fp and the electron acceptor/donor component in the membrane is not known in detail [10,11]. Structural information on how the FAD and the iron-sulfur centers are arranged within the enzyme is required in order to understand fully the assembly and the function of SDH and FRD.

The complete amino acid sequence of Ip is available from *E. coli* FRD [12] and SDH [13], *Bacillus subtilis* SDH [14], bovine heart SDH [15] and *Proteus vulgaris* FRD [16]. They contain 11 (*E. coli* SDH only 10) cysteine residues at conserved positions distributed in three clusters designated I, II and III [1,13]. Based on amino acid sequence comparisons with less complex iron-sulfur proteins of known three-dimensional structure, it has been proposed that the four cysteines of cluster I are ligands to the 2Fe center, whereas clusters II and III would ligate the 3Fe and 4Fe centers [1,17]. Herein we present experimental evidence from *B. subtilis* mutant SDH for the ligation of the 2Fe center to the cysteine residues of cluster I.

The 2Fe, 3Fe and 4Fe centers have been demonstrated in *B. subtilis* SDH and are called S-1, S-3 and S-2, respectively [18,19]. EPR spectroscopy of *B. subtilis* mutants defective in Ip showed that center S-1 can be assembled in soluble SDH, provided that the mutant contained Fp and a fragment of about 15 kDa or larger of Ip [18]. Wild-type Ip has a molecular mass of 28 kDa. From these previous results it could not be unam-

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biguously concluded whether the Ip fragments in the mutants were truncated polypeptides resulting from nonsense mutations or were proteolytic degradation products from a full-length mutant Ip. We have now analysed Ip polypeptides from four mutants, mapped the respective mutations in the structural gene for Ip, *sdhB*, and identified one mutation by DNA sequence analysis. The results together with previous EPR data show that SDH with a truncated Ip lacking cysteine clusters II and III can still ligate iron-sulfur center S-1.

2. MATERIALS AND METHODS

2.1. *Bacterial strains, plasmids and phages*

The origin and Sdh phenotype of *B. subtilis* mutants KA94109 (*sdhC109, ilvB2*), KA97083 (*sdhB83, trpC2, leu-2*), KA99103 (*sdhB103, leu-2*), KA97118 (*sdhB118, trpC2, leu-2*) and KA99123 (*sdhB123, leu-2*) have been described in [20]. KA99103 and KA99123 are Trp^+ derivatives of strains KA97103 and KA97123. The *B. subtilis sdhC, sdhA* and *sdhB* genes encoding cytochrome *b*-558, Fp and Ip were until 1987 designated *sdhA, sdhB* and *sdhC*, respectively [14]. *E. coli* JM83 (*ara, Δ(lac-proAB), rpsL, φ80, lacZΔ*M15) [21] and 5K (*hsdR, thi, thr, rpsL, lacZ*) [22] were used for plasmid production. *E. coli* JM101 (*supE, thi, Δ(lac-proAB)*, [F′, *traD36, proAB, lacI^{q}ZΔ*M15]) and *E. coli* JM103 (*thi, rpsL, sbcB15, endA, hspR4, supE, Δ(lac-proAB)*, [F′, *traD36, proAB, lacI^{q}ZΔ*M15]) were the hosts for propagation of bacteriophages M13 mp18 and M13 mp19 and recombinants thereof [21].

Plasmids pSH1047 (*sdhC sdhA sdhB gerE*) [23], pSDHΔ12 (*sdhB*) [24] and pUC18 and pUC19 [21] have been described before. pLUW1 is pUC18 into which the 4.5 kbp *sdhAB* *Pst*I fragment of pSH1047 has been inserted. Integration vector pLUW3 is a 6.3 kbp derivative of pNO1523 [25] containing the *kan* gene of pUB110 [26] and a 395 bp *Hin*dIII-*Pvu*II *B. subtilis* DNA fragment (ORF2) from the region just downstream of *sdhB* (fig.2).

2.2. *Growth media and transformation*

E. coli JM strains and strain 5K were made competent by $CaCl_2$ treatment [27] and by the method of Hanahan [28], respectively. *B. subtilis* was grown to competence as described by Arwert and Venema [29], except that the cells were grown at 37°C for 4.5 instead of 3.5 h. *E. coli* transformants were selected on LA plates with ampicillin (35 mg/l). *B. subtilis* kanamycin-resistant transformants were selected on tryptose blood agar base (Difco, USA) plates containing kanamycin sulfate (5 mg/l). Sdh^+ transformants were selected on minimal citrate/glutamate plates [30].

2.3. *In vitro DNA techniques*

Plasmid DNA was isolated according to the procedure of Ish-Horowitz and Burke [31]. *B. subtilis* chromosomal DNA was isolated as described for plasmid DNA by Canosi et al. [32]. Restriction endonuclease digestions, agarose gel electrophoresis and ligation with bacteriophage T_4 DNA ligase followed standard methods [33]. DNA fragments were generally isolated from agarose gels using Geneclean (BIO 101, USA). Propagation of bacteriophage M13 and isolation of single-stranded DNA were carried out according to Messing [34]. DNA sequence analyses were performed using the dideoxy chain-termination method [35] with modified T_7 DNA polymerase [36].

3. RESULTS

3.1. *IP antigen in mutants*

B. subtilis mutants KA97083, KA99103, KA97118 and KA99123 lack SDH activity, have normal Fp subunits in the cytoplasm and are defective in Ip due to mutations in the *sdhB* gene [20]. With the exception of KA99123, they contain iron-sulfur center S-1 as determined by EPR spectroscopy of cell-free extracts [18]. The mutants and a reference strain KA94109 (mutant with cytoplasmic wild-type Fp and Ip subunits) were analysed with respect to the Ip subunit. Extracts were immunoadsorbed with anti-Ip antibodies and the antigens were then run in SDS-polyacrylamide gel electrophoresis (fig.1).

The wild-type Ip from KA94109 appeared as a doublet band with an apparent molecular mass of 28 kDa. Mutants KA99103 and KA97118 contained a distinct single Ip antigen of approx. 16 kDa (fig.1, lanes B,C), whereas KA97083 had an antigen of about 19 kDa (lane E). Strain KA99123 lacked detectable Ip (lane D). Full-length Ip was not found in any of the mutants. A trace of 28 kDa Ip antigen has however been detected in KA99123 cell extracts using immunoblot analysis (Schröder, I., unpublished).

3.2. *Genetic mapping of* sdhB *mutations*

The *sdhB* mutation in each mutant was mapped by transformation with overlapping wild-type *B. subtilis* DNA fragments corresponding to the *sdhB* region of the chromosome. The DNA fragments used and the results of the experiments are shown in fig.2.

Mutations *sdhB103* and *sdhB118* which both result in an about 16 kDa Ip antigen, mapped to a 200 bp *Dra*I-*Hin*dIII fragment from the middle part of *sdhB*. The two mutations are possibly identical. Mutation *sdhB83* has previously been mapped to a location distal to *sdhB103* [20] and upstream of the *Nar*I site at nucleotide 3245 in *sdhB* [37]. The *Hin*dIII fragment of subclone pSDHB45 carried the wild-type allele of *sdhB83*.

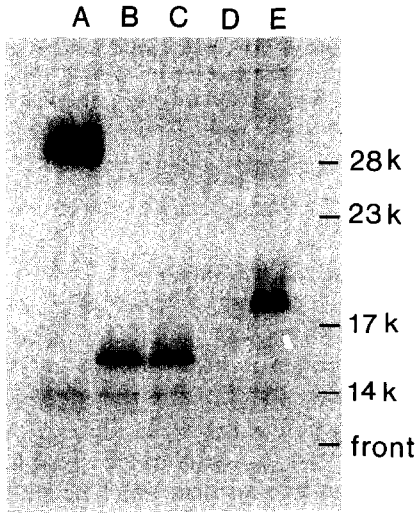

Fig.1. Ip antigens immunoadsorbed from [^{35}S]methionine-labeled mutant *B. subtilis* cell lysates and analyzed by SDS-polyacrylamide (10–15% acrylamide) gel electrophoresis. An autoradiograph of the gel is shown. Preparation of cell lysates, immunoadsorption using protein A-Sepharose 4B-Cl and electrophoresis were performed as in [14]. A, KA94109 (wild-type Ip); B, KA99103 (*sdhB103*); C, KA97118 (*sdhB118*); D, KA99123 (*sdhB123*); E, KA97083 (*sdhB83*).

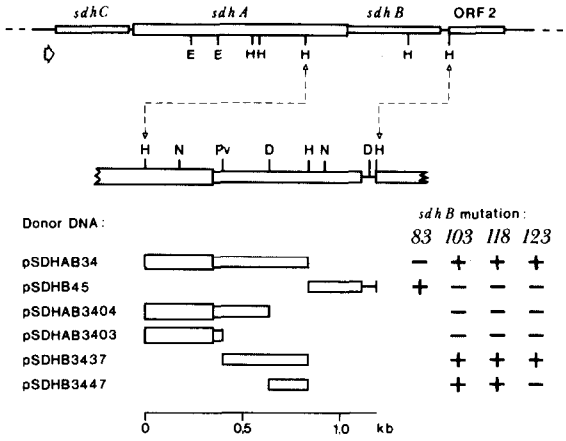

Fig.2. Map of the *sdh* region in *B. subtilis* and mapping of *sdhB* mutations. The DNA fragments shown in the lower part were isolated from pLUW1 or pSDHΔ12 and cloned into pUC18 or pUC19. *B. subtilis* with the indicated *sdhB* mutations was then transformed with saturating amounts of the purified plasmids and Sdh$^+$ transformants were selected (+, transformants; −, no transformants). Relevant endonuclease restriction sites are indicated; D, *Dra*I; E, *Eco*RI; H, *Hin*dIII; N, *Nar*I; Pv, *Pvu*II. The short arrow just before *sdhC* shows the start-point and direction of transcription of the *sdh* operon.

From these results we conclude that *sdhB83* is located on an 86 bp fragment flanked by nucleotides 3159 and 3245. Mutation *sdhB123* seems to be located close to the *Dra*I site in *sdhB*, because it could only be rescued by DNA with this site intact. Alternatively, *sdhB123* is two mutations, one on each side of the *Dra*I site (fig.2).

3.3. sdhB103 *is a nonsense mutation*

The location of mutation *sdhB103* in the structural gene for the Ip polypeptide and the size of the resulting Ip antigen strongly suggested that a stop codon has been introduced, leading to a truncated gene product. To confirm this and to map the exact location of the mutation we cloned *sdhB* from KA99103 and determined the nucleotide sequence of the mutated DNA fragment. The cloning was performed with the use of integration vector pLUW3 essentially by the same technique as described previously for the isolation of *sdhC* mutant genes [30]. Plasmid pSDHB103 was generated from *Eco*RI-digested KA99103::pLUW3 chromosomal DNA. The plasmid contained the mutant *sdhB* gene and 1 kbp of *sdhA*.

The 200 bp *Dra*I-*Hin*dIII fragment (see fig.2) of pSDHB103 was subcloned in phage M13 and the complete nucleotide sequence of both strands was determined. Only one base pair change was found compared to the wild-type sequence [14]. The G to A substitution at nucleotide 3113 changes a tryptophan codon to an UGA stop codon. The predicted product from this mutant *sdhB* gene is a truncated Ip polypeptide, 146 amino acid residues long and with a calculated mass of 16841 Da, the initiation methionine excluded [38].

4. DISCUSSION

Iron-sulfur centers in proteins are usually ligated to cysteine residues, however, a non-cysteine residue like histidine can also function as a ligand [17,39]. The 2Fe center in succinate oxidoreductases is most likely ligated to four cysteine residues in the Ip subunit.

A successful separation of SDH into Fp and Ip without destruction of the center has not been reported. The ligands to the 2Fe center S-1 have

tentatively been identified from the following observations: (i) Fp does not contain cysteine residues that are conserved in all succinate oxidoreductases [1,14]. (ii) The 2Fe center of *Wolinella (Vibrio) succinogenes* FRD is associated with the 31 kDa subunit as determined by EPR spectroscopy of the resolved enzyme [40]. (iii) *B. subtilis* mutant KA99123 (*sdhB123*) contains functional Fp but lacks detectable amounts of Ip and the center S-1 [18]. (iv) The ligands to center S-1 in mammalian SDH are, as determined by the electron spin echo envelope modulation technique, probably four cysteine residues [41]. (v) The spacing between the four conserved cysteines of cluster I in Ip is, as pointed out by several authors, very similar to that of the ligands to 2Fe centers in plant-type ferredoxins [1,17].

B. subtilis mutant KA99103 (*sdhB103*) contains center S-1 and was shown in this work to synthesize a truncated Ip polypeptide of 16.8 kDa. The shortened Ip has only four cysteine residues and lacks histidine residues. These experimental findings combined with the observations listed above strongly suggest that the [2Fe-2S] center in *B. subtilis* SDH is ligated to the Ip polypeptide by the cysteine residues at positions 63, 68, 71 and 83 (fig.3).

Mutation *sdhB83* is probably also a nonsense mutation, since it maps in a sequence of *sdhB* encoding amino acid residues 163–191 and cell lysates of KA97083 contain an Ip fragment of about 19 kDa (fig.1). The size of the fragment corresponds to a polypeptide of about 180 residues. This truncated Ip would contain all the cysteine residues of cluster I and probably all four cysteine residues of cluster II (fig.3). These properties of KA97083 and the fact that center S-1 is present in this mutant can be useful in studies on the role of the cluster II cysteines in ligation of centers S-2 and S-3.

Studies on *B. subtilis* SDH-defective mutants indicate that Ip in the cytoplasm is stabilized by association with Fp [18]. In the absence of functional Fp, the Ip polypeptide (wild-type and mutant alike) is rapidly degraded in cell lysates. Mutants KA99103 and KA97083 contain, in contrast to KA99123, relatively stable Ip fragments with center S-1 incorporated. Apparently, the N-terminal 146 residues of Ip can separately form a domain which contains only one of the three iron-sulfur centers of SDH and is still able to associate with Fp. However, Fp associated with this domain of Ip cannot be bound to cytochrome *b*-558, the anchor protein and electron acceptor for SDH in the *B. subtilis* cytoplasmic membrane [30]. Center S-3 is most likely ligated to cysteine residues in the C-terminal half of Ip. An intact center S-3 is required for membrane binding of mammalian SDH [42]. Seemingly, the C-terminal part of Ip contains (part of) the structures that specifically can recognize and bind to the membrane anchor protein(s).

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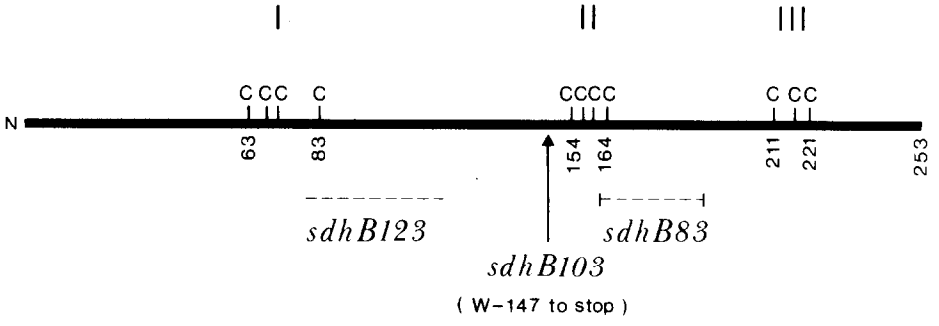

Fig.3. Schematic illustration of the *B. subtilis* Ip polypeptide showing the location of cysteine residues, mapped mutations and the effect of nonsense mutation *sdhB103*. The four conserved cysteine residues of cluster I are concluded to be the ligands to the [2Fe-2S] center in SDH.

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