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Processing of *Bacillus subtilis* succinate dehydrogenase and cytochrome *b*-558 polypeptides

Lack of covalently bound flavin in the *Bacillus* enzyme expressed in *Escherichia coli*

Lars Hederstedt*, Tomas Bergman and Hans Jörnvall

**Department of Microbiology, University of Lund, Sölvegatan 21, S-223 62 Lund* and *Department of Chemistry I, Karolinska Institutet, S-104 01 Stockholm, Sweden*

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The DNA sequence of the *Bacillus subtilis sdh* operon coding for the two succinate dehydrogenase subunits and cytochrome *b*-558 (the membrane anchor protein) has recently been established. We have now determined the extent of N-terminal processing of each polypeptide by radiosequence analysis. At the same time, direct evidence for the correctness of the predicted reading frames has been obtained. The cytochrome showed a ragged N-terminus, with forms lacking one residue, and is inserted across the membrane without an N-terminal leader-peptide. Covalently bound flavin was not detectable in *B. subtilis* succinate dehydrogenase expressed in *Escherichia coli* despite normal N-terminal processing of the apoprotein. This provides an explanation to why the succinate dehydrogenase synthesized in *E. coli* is not functional and demonstrates that host-specific factors regulate the coenzyme attachment.

Succinate dehydrogenase; Post-translational processing; Heterologous system; Electroblotting; Radiosequence analysis; N-terminal heterogeneity

1. INTRODUCTION

Succinate dehydrogenase (SDH) (EC 1.3.99.1) consists of two protein subunits, a larger flavoprotein (Fp) with one 8α-N(3)His-bound FAD, and a smaller iron-sulfur protein (Ip) containing three different iron-sulfur centers [1,2]. It is not known by what mechanism(s) flavin becomes covalently bound to this or other proteins [3–5].

Bacillus subtilis SDH can be solubilized from the cytoplasmic membrane as a complex consisting of equimolar amounts of Fp (65 kDa), Ip (28 kDa) and cytochrome *b*-558 (23 kDa) [6]. The cytochrome spans the lipid bilayer and anchors SDH to the inner surface of the cytoplasmic membrane [7]. The nucleotide sequence of the genes coding for Fp, Ip, and cytochrome *b*-558 was recently determined from the cloned *B. subtilis sdhCAB* operon [8,9]. To confirm each of the reading frames predicted from the DNA sequence and to determine possible N-terminal post-translational processings of the subunits, we have studied the proteins by radiosequence analysis. The cytochrome was of particular interest, since it could be synthesized as a larger precursor with an N-terminal leader peptide, similar to those which have been found in other proteins that are transported across membranes [10].

All three polypeptides of the *B. subtilis* SDH–cytochrome *b*-558 complex are expressed in *Escherichia coli* from the *sdhCAB* operon cloned in plasmid vectors [9,11,12]. However, the

Correspondence address: L. Hederstedt, Dept of Microbiology, University of Lund, Sölvegatan 21, S-223 62 Lund, Sweden

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heterologous enzyme complex is not assembled on the membrane. We show here that *B. subtilis* Fp synthesized from the intact *sdhA* gene in *E. coli* does not contain covalently bound flavin. This finding suggests a structural difference in apo-Fp synthesized in the two bacteria or an absence in *E. coli* of special factors required for flavinylation of *B. subtilis* SDH.

2. MATERIALS AND METHODS

2.1. *Bacterial strains and growth*

B. subtilis BR95 (*trpC2 ilvC1 pheA1*) was grown in minimal medium [6] with 1% (w/v) sodium succinate as a source of carbon and energy. The medium was supplemented with 0.12% (w/v) amino acids (casamino acids, Difco), 10 μM $MnCl_2$, 4 mCi/l L-[2,3,4,5,6-^{3}H]Phe (0.13 Ci/mol, Amersham), 20 mg/l L-Trp, 20 mg/l L-Ile and 20 mg/l L-Val. *B. subtilis* KA97123 (*trpC2 leu-2 sdhB123*) was grown in nutrient sporulation medium [6], pH 7, without added $MnCl_2$ and containing 2 mCi/l [^{35}S]Met (1.42 Ci/mol, Amersham). *E. coli* MV10Ch3/86 (C600 *ΔtrpE5*::Ch3/86) containing plasmid pSH1047 which carries the cloned *B. subtilis sdhCAB* operon [9,13] was grown in nutrient sporulation medium, pH 7, supplemented with 0.5% (w/v) glucose, 20 mg/l thiamine, 40 mg/l nicotinic acid, 20 mg/l L-Trp, 50 mg/l kanamycin (Sigma) and 2.2 mCi/l [^{35}S]Met. The bacteria, in about 300 ml medium, were grown at 37°C in 2.8 l indentated Fernbach flasks on a rotary shaker (200 rpm) and were harvested in the late exponential growth phase. *B. subtilis* membranes and protoplasts were prepared as described [6]. *E. coli* spheroplasts were prepared in 20% (w/v) sucrose, 30 mM Tris-HCl, pH 8, as described [14].

2.2. *Isolation of subunits*

Antibodies against the *B. subtilis* Fp subunit [15] were used for test-tube precipitation [16] of solubilized SDH–cytochrome *b*-558 complex from [^{3}H]Phe-labelled *B. subtilis* BR95 membranes and of soluble Fp from [^{35}S]Met-labelled cells. The soluble cell fractions were prepared from protoplasts of *B. subtilis* KA97123 and from spheroplasts of *E. coli* MV10Ch3/86(pSH1047). The protoplasts/spheroplasts from 225–250 ml cultures (A_{600} = 0.7) were collected by centrifugation (7000 × *g*, 20 min, 15°C) and were lysed in 1.5 ml cold 30 mM Tris-HCl, pH 8.0, containing 10 mM Na-EDTA, 2 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine-HCl, and 5 mg/l each of chymostatin, leupeptin, elastatinal and antipain (Sigma). The cell lysates were gently sonicated and centrifuged (45000 × *g*, 30 min, 4°C). The supernatant was centrifuged (100000 × *g*, 1 h, 4°C) to produce the soluble cell fraction.

2.3. *Analytical methods*

Liquid-phase amino acid sequence analysis of immunoprecipitates was performed with a Beckman 890D sequencer. Gas-phase sequencer degradations were carried out in an Applied Biosystems 470A instrument with polypeptides separately electroblotted from SDS polyacrylamide gels directly onto polybrene-coated glass-fiber filter discs [17]. Before sequencer applications, apomyoglobin was added as carrier and standard. Phenylthiohydantoin derivatives from degradations of the myoglobin were identified in aliquots by reverse-phase high-performance liquid chromatography [18], the remaining parts being used for determinations of radioactivity by liquid scintillation counting. Protein was determined by the procedure of Lowry et al. [19] in the presence of 1.7% (w/v) SDS and with serum albumin as standard. Fluorimetric determination of covalently bound flavin [20], SDS-polyacrylamide gel electrophoresis, and autoradiography were as described [21].

3. RESULTS AND DISCUSSION

3.1. *N-terminal structure of Fp, Ip and cytochrome*

[^{3}H]Phe-labelled *B. subtilis* SDH–cytochrome *b*-558 complex was isolated from Triton X-100-solubilized membranes by immunoprecipitation using anti-Fp antiserum. The precipitate contained equimolar amounts of Fp, Ip and cytochrome *b*-558 polypeptides apart from immunoglobulins, and was free from other radioactive bacterial proteins (fig.1A). The immunoprecipitate was mixed with a 30-fold excess of apomyoglobin (10 nmol) added as carrier and internal standard and was submitted to radiosequence analysis. The results are shown in fig.2. The immunoglobulin chains did not interfere with the analysis.

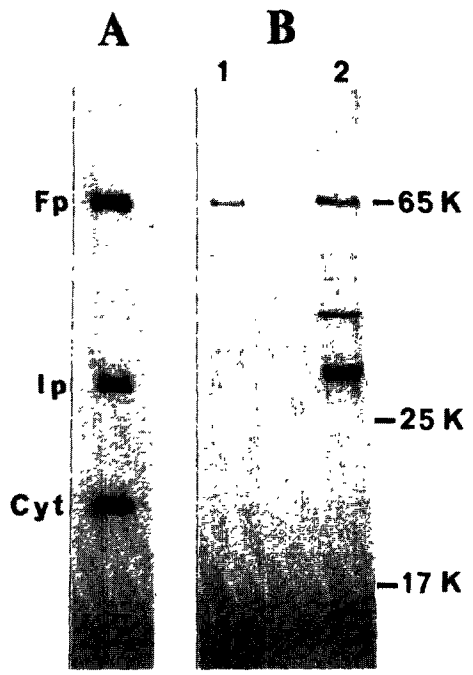

Fig.1. [^{3}H]Phe-labelled SDH–cytochrome *b*-558 complex (A) and [^{35}S]Met-labelled Fp from *E. coli* (pSH1047) (B, lane 1) and from *B. subtilis* KA97123 (B, lane 2) immunoprecipitated with anti-Fp antiserum and analyzed by SDS-polyacrylamide gel electrophoresis (10–15% gradient gel in A and 12% uniform gel in B). Autoradiographs (fluorograph in A) are shown. Fp, Ip, and cytochrome *b*-558 contain 20, 9 and 20 Phe residues per polypeptide, respectively [8,9]. The bands seen at a molecular mass smaller than 65 kDa in panel B are breakdown fragments of Fp.

Major peaks of ^{3}H were obtained in cycles 8, 25, and 31 (derived from Ip + cytochrome, Ip, and Fp, respectively, as shown in fig.3), minor peaks in cycles 7 and 24 (both from shorter forms of cytochrome, cf. fig.3). To resolve the overlapping degradation patterns, each radioactive subunit was separately electroblotted from a gel after preparative SDS-polyacrylamide electrophoresis, after which apomyoglobin was added. The results of degradation of the filter-immobilized proteins are shown in fig.4. Ip contained the first ^{3}H-labelled Phe at position 8 in agreement with the results obtained from analysis of the whole complex. Cytochrome *b*-558 contained ^{3}H-labelled Phe at positions 6, 7, 8, 9, 23 and 24. These results also agree with those in fig.2 and indicate the presence

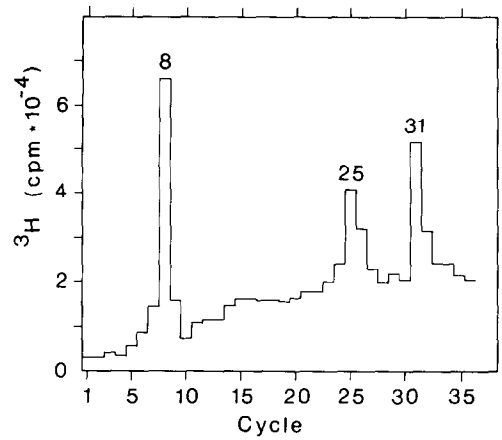

Fig.2. N-terminal radiosequence analysis of [^{3}H]Phe-labelled SDH–cytochrome complex isolated from *B. subtilis* membranes. The N-terminal amino acid sequence of each polypeptide as deduced from the DNA sequence and the radiosequence analysis of the proteins is shown in fig.3.

of a ragged N-terminus derived from the presence/absence of one residue. Thus, the cytochrome isolated from the membrane-bound functional complex apparently has two forms in different amounts (fig.3). Similar microheterogeneities have been found in other proteins [22].

We conclude from the N-terminal analysis of [^{3}H]Phe-labelled subunits that: (i) the open reading frame and the initiation of translation predicted from the DNA sequence are correct for all three polypeptides; and that (ii) the Met coded for by the AUG initiation codon has been removed from the native Fp and Ip, and non-stoichiometrically (to about 50%) from the native cytochrome *b*-558 polypeptides (fig.3).

It is unknown by what mechanism the cytochrome *b*-558 is inserted across the cytoplasmic membrane. The degradation data in relation to the DNA sequence show that the protein is not translated as a larger precursor with an N-terminal extension which is cleaved off upon integration into the membrane. The lack of a larger precursor is also supported by the observation that the original N-terminal sequence deduced from the DNA lacks the characteristics of a leader sequence [8]. The molecular mass of the cytochrome *b*-558 polypeptide as estimated from SDS-polyacrylamide gel electrophoresis and as calculated from the nucleotide sequence [8] differs by 4 kDa, but

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          1    5    10   15   20   25   30   35
Fp        SQSSIIVVGGGLAGLMATIKAAESGMAVKLFSIVPVK
Ip        SEQKTIRFIITRQDTADSTPYDEEFEIPYRPNLNVIS
Cyt  55%  MSGNREFYFRRLHSLLGVIPVGIFLIQHLVVNQFAAR
     45%  SGNREFYFRRLHSLLGVIPVGIFLIQHLVVNQFAARG
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Fig.3. N-terminal amino acid sequence of *B. subtilis* Fp, Ip, and cytochrome *b*-558 polypeptides. The structures are as determined by DNA sequence analysis and the starting points as established from the radiosequence analysis (Phe (F) labelled is underlined). Two forms of cytochrome *b*-558 polypeptide were found.

this is likely to be within the accuracy of the estimate from electrophoresis (cf. [23,24]).

3.2. B. subtilis *Fp expressed in* E. coli *is defective*

The three polypeptides of the *B. subtilis* SDH–cytochrome *b*-558 complex are expressed from plasmid pSH1047 in *E. coli*, but the functional complex is not assembled [9]. Fp and Ip are found in the cytoplasm, whereas cytochrome *b*-558 with normal physico-chemical properties is found in the inner membrane. Significantly, active membrane-bound SDH complex is expressed from pS1047 in *B. subtilis* [12]. These facts demonstrate that the *sdhCAB* operon in pSH1047 is intact and suggest that an answer to why the complex is not formed in *E. coli* (pSH1047) is to be found in differences in translational/post-translational processing of the SDH subunits in the two bacteria.

A slightly faster electrophoretic mobility of *B. subtilis* Fp synthesized in *E. coli* compared to that of *B. subtilis* indicated a defect ([9] and fig.1B). The Fp in *B. subtilis* undergoes at least two post-translational modifications before assembly into the SDH complex; the N-terminal Met is removed (this work) and the His at position 40 is flavinylated [25]. To determine if these two modifications also occur in *E. coli* we isolated [^{35}S]Met-labelled Fp by immunoprecipitation from *E. coli* MV10Ch3/86(pSH1047) and as a control from *B. subtilis* mutant KA97123. This mutant lacks Ip subunits and therefore accumulates flavinylated and reconstitutively active Fp in the cytoplasm [21]. Each immunoprecipitated protein (fig.1B) was analyzed for covalently bound flavin and for amino acid sequence by 30 cycles of radiosequence degradation.

The *B. subtilis* Fp from both *E. coli* and *B. subtilis* has the first [^{35}S]Met at position 16 and the second at 26 (not shown). These results complement and confirm the [^{3}H]Phe sequence data (fig.3). Furthermore, they show that the Fp from the two bacteria is processed identically at the N-terminus which contains the $\beta\alpha\beta$ fold [9,26] important for FAD-protein interaction.

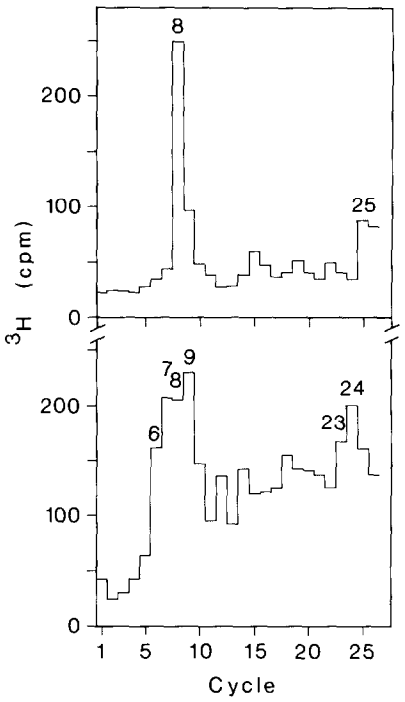

Fig.4. N-terminal radiosequence analysis of isolated and electroblotted [^{3}H]Phe-labelled Ip (top panel) and cytochrome *b*-558 (bottom panel) polypeptides from *B. subtilis* SDH complex.

The content of covalently bound flavin in the Fp isolated from *E. coli* (pSH1047) was less than 15%, i.e. not detectable, compared to that of Fp synthesized in *B. subtilis* (table 1). This deficiency does not seem to result from a general disturbance of the flavinylating machinery in the *E. coli* cell. Membranes from *E. coli* (pSH1047) and from cells containing the plasmid vector without *sdh* DNA have approximately the same amount of membrane-bound SDH activity [9]. Thus, the biosynthesis of homologous SDH in *E. coli* is not affected by the simultaneous synthesis of relatively large amounts of *B. subtilis* SDH protein. Furthermore, fumarate reductase amplified 30–40-fold in aerobically grown *E. coli* is fully flavinylated [27].

Table 1

Flavin content in *B. subtilis* [^{35}S]Met-labelled Fp isolated by immunoprecipitation from the soluble cell fraction of *B. subtilis* and *E. coli* strains

Source of Fp antigen	Covalently bound flavin/Fp antigen	
	Determined (nmol/cpm $\times 10^{-6}$)[a]	Estimated (nmol/mg protein)[b]
B. subtilis KA97123	1.2	5.7
E. coli MVCh3/86(pSH1047)	<0.2	<0.8

[a] The specific activity in Fp from *B. subtilis* and *E. coli* was approximately the same as judged from autoradiographs of crossed immunoelectrophoresis plates containing identical amounts of antigen

[b] ^{35}S content per protein precipitable with trichloroacetic acid from the *B. subtilis* and *E. coli* soluble cell fractions was identical. Fp antigen in the immunoprecipitates was calculated assuming 4.1 mol% Met in both Fp and the average soluble bacterial protein

SDH and fumarate reductase are very similar and contain the same prosthetic groups [2].

The factors needed for the covalent attachment of FAD to Fp in *B. subtilis* are unknown. Ip or cytochrome *b*-558 subunits are not involved, and mutations affecting the flavinylation have only been found within the structural gene for Fp [21,25]. Folding of apo-Fp seems necessary before the cofactor can be bound; e.g. the N-terminal 150 amino acid residues are not sufficient for the flavinylation of His-40 to occur, and different amino acid substitutions far downstream from residue 100 can prevent the covalent modification [25].

The flavin deficiency in *B. subtilis* Fp expressed in *E. coli* together with a normal assembly of *E. coli* SDH suggest that host-specific factors can either be required for or prevent the flavinylation. This is in contrast to what has been found for *Arthrobacter oxidans* 6-hydroxy-D-nicotine oxidase which also contains 8α-N(3)His-bound FAD. The *A. oxidans* enzyme expressed in *E. coli* in vivo and in vitro is flavinylated and functional [28]. On the other hand, attempts to flavinylate *E. coli* fumarate reductase in vitro have been reported to be unsuccessful [29].

The *B. subtilis* apo-Fps synthesized in *E. coli* and *B. subtilis*, respectively, are possibly not structurally identical although the N-termini are the same. This could cause the flavin defect and is indicated by three independent observations. First, the increased electrophoretic mobility of Fp isolated from *E. coli* is not unambiguously explained by the absence of flavin. Different *B. subtilis* mutant Fp subunits which lack flavin as a result of single amino acid substitutions co-migrate or migrate slower than the wild-type subunit ([21] and Hederstedt, unpublished). Second, the SDH–cytochrome *b*-558 complex with a full complement of iron-sulfur centers can be assembled in *B. subtilis* without the flavin [25,30]. Third, the iron-sulfur center S-1 is not detectable in the *B. subtilis* SDH protein present in *E. coli* (pSH1047) cell lysates (Andersson and Hederstedt, preliminary EPR data). Similarly, mutants of *B. subtilis* mutated in Fp such that SDH is not assembled also lack flavin in Fp as well as center S-1 [21,30].

Our finding that *B. subtilis* Fp synthesized in *E. coli* lacks the prosthetic group has important implications for research aimed at identifying factors needed for the covalent attachment of flavin to proteins. The apo-Fp made in *E. coli* can be produced in large quantities and used as substrate in in vitro studies of flavinylation. Furthermore, if a single *B. subtilis* gene product is required for the modification it would be possible to clone that gene in *E. coli* (pSH1047) by selecting for *B. subtilis* SDH activity in an *E. coli* SDH negative background. Alternatively, if an *E. coli* gene product prevents the flavinylation it may be possible to isolate mutants defective in that product.

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