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Analysis of covalent flavinylation using thermostable succinate dehydrogenase from *Thermus thermophilus* and *Sulfolobus tokodaii* lacking SdhE homologs

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A B S T R A C T

Recent studies have indicated that post-translational flavinylation of succinate dehydrogenase subunit A (SdhA) in eukaryotes and bacteria require the chaperone-like proteins Sdh5 and SdhE, respectively. How does covalent flavinylation occur in prokaryotes, which lack SdhE homologs? In this study, I showed that covalent flavinylation in two hyperthermophilic bacteria/archaea lacking SdhE, *Thermus thermophilus* and *Sulfolobus tokodaii*, requires heat and dicarboxylic acid. These thermophilic bacteria/archaea inhabit hot environments and are said to be genetically far removed from mesophilic bacteria which possess SdhE. Since mesophilic bacteria have been effective at covalent bonding in temperate environments, they may have caused the evolution of SdhE.

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1. Introduction

Flavoproteins constitute a large family of enzymes involved in major redox reactions, including oxidative phosphorylation and the metabolism of sugars, amino acids, and fatty acids. Among the flavoproteins, succinate dehydrogenase (SDH) is conserved throughout all domains of life, and is the only enzyme supporting the tricarboxylic acid cycle and electron transport chain. SDH catalyzes the oxidization of succinate into fumarate using flavin adenine dinucleotide (FAD) as a cofactor. In eukaryal and many mesophilic bacterial enzymes, SDH consists of four subunits, comprising a flavoprotein subunit (SdhA), an iron–sulfur subunit (SdhB), and two membrane anchor subunits (SdhC and SdhD). SdhA covalently links to FAD [1,2], creating a dicarboxylate active site [3,4]. This covalent flavinylation is a post-translational self-catalytic process, in which the isoalloxazine ring at the 8α-carbon of FAD is linked to a highly conserved histidine residue, such as His44 in the fumarate reductase (FRD) of *Escherichia coli* [5–11].

The mitochondrial protein Sdh5 is required for covalent flavinylation of FAD to SdhA in yeast [12–16]. Similarly, succinate dehydrogenase protein E (SdhE) binds directly to FAD and is required for the covalent flavinylation of SdhA in bacteria. SdhE homologs exist in α-, β-, and γ-proteobacteria. SdhE evolved once in ancestral α-proteobacteria [17,18].

However, the mechanism underlying covalent flavinylation in their ancestors—such as thermophile prokaryotes, which lack SdhE homologs—remains unclear.

In this study, I analyzed SDH flavoprotein subunit A (SdhA) in the thermophiles *Thermus thermophilus* and *Sulfolobus tokodaii*. The SDH enzyme in these two hyperthermophilic species consists of four subunits that resemble those in mesophilic species. These hyperthermophiles lack SdhE, so the question of how covalent flavinylation of SdhA occurs is significant. I purified thermostable recombinant SdhA protein for analyses of flavinylation. In this paper, I present conditions for the covalent flavinylation of SdhA in *T. thermophilus* and *S. tokodaii* and discuss the evolutionary emergence of SdhE for mediating this reaction.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The *E. coli* strain JM109 (Takara Bio Inc., Otsu, Shiga, Japan) used for cloning was grown in lysogeny broth (LB) containing 50 μg/ml kanamycin. The *E. coli* BL21-CodonPlus® (DE3)-RIL strain (Stratagene Corporation, La Jolla, CA, USA) used for SdhA expression analysis was grown in LB containing 50 μg/ml kanamycin.

2.2. Plasmid construction

The plasmid pET28a [Novagen (EMD) Biosciences, Inc., Madison, WI, USA] was used in cloning, sequencing, and expression experiments. The *T. thermophilus* SdhA gene encoding the SdhA protein (UniProt accession code: Q5SIC0) was amplified by polymerase chain reaction (PCR) from the genomic DNA of *T. thermophilus* strain HB8 (Takara Bio Inc.); the *S. tokodaii* SdhA gene encoding the SdhA protein (UniProt accession code: Q9C4L9) was amplified from the genomic DNA of *S. tokodaii* strain 7; and the *E. coli* SdhE gene encoding the SdhE protein (UniProt accession code: P64559) was amplified from the genomic DNA of *E. coli* strain K-12 W3110, using oligonucleotide primers based on the genomic DNA sequence (Supplementary Table S1). The predicted *T. thermophilus* SdhA (TthSdhA) PCR product of 1734 base pairs (bp), *S. tokodaii* SdhA (StoSdhA) PCR product of 1701 bp, and *E. coli* (EcoSdhE) PCR product of 267 bp were subcloned into a pET28a vector containing an N-terminal hexahistidine tag (Fig. 1A). The resulting expression vectors, TthSdhA-pET28a, StoSdhA-pET28a, and EcoSdhE-pET28a, were transformed into *E. coli* strain JM109 competent cells for sequencing. The sequences of SdhA and SdhE were verified using the dideoxy chain termination method, with a vector-specific T7 promoter/T7 terminator, on an automatic DNA sequencer (ABI Prism® 310A Genetic Analyzer; Applied Biosystems, Inc., Carlsbad, CA, USA).

2.3. Expression and purification of hexahistidine-tagged recombinant of Thermus thermophilus and Sulfolobus tokodaii succinate dehydrogenase subunit A and Escherichia coli succinate dehydrogenase protein E

The vectors TthSdhA-pET28a, StoSdhA-pET28a, and EcoSdhE-pET28a were transformed into host *E. coli* BL21-CodonPlus® (DE3)-RIL cells. The *E. coli* transformants were grown overnight at 25 °C on a shaker [250 revolutions per minute (rpm)] in LB containing 50 μg/ml kanamycin. Next, the recombinant proteins were overproduced over 24 h at 25 °C by induction with 1 mM isopropyl β-D-1-thiogalactopyranoside. The cells were pelleted by centrifugation and stored at −80 °C until further use. For purification, the *E. coli* cells were thawed; suspended in 20 mM potassium phosphate buffer (pH 7.0) containing 300 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride (1X), ethylenediaminetetraacetic acid-free Complete™ Protease Inhibitor Cocktail (Roche Diagnostics, Basel, Switzerland), and 10 mM imidazole; and disrupted by brief sonication on ice. The lysate was pelleted by ultracentrifugation (RP50T ultracentrifuge/P50AT2-750 rotor; Hitachi Ltd., Tokyo, Japan; 30 000 rpm) for 30 min at 4 °C. Hexahistidine-tagged recombinant TthSdhA, StoSdhA and SdhE in the supernatant were purified through nickel-nitrilotriacetic acid Superflow columns (Qiagen GmbH, Hilden, Germany) at 4 °C. The columns were
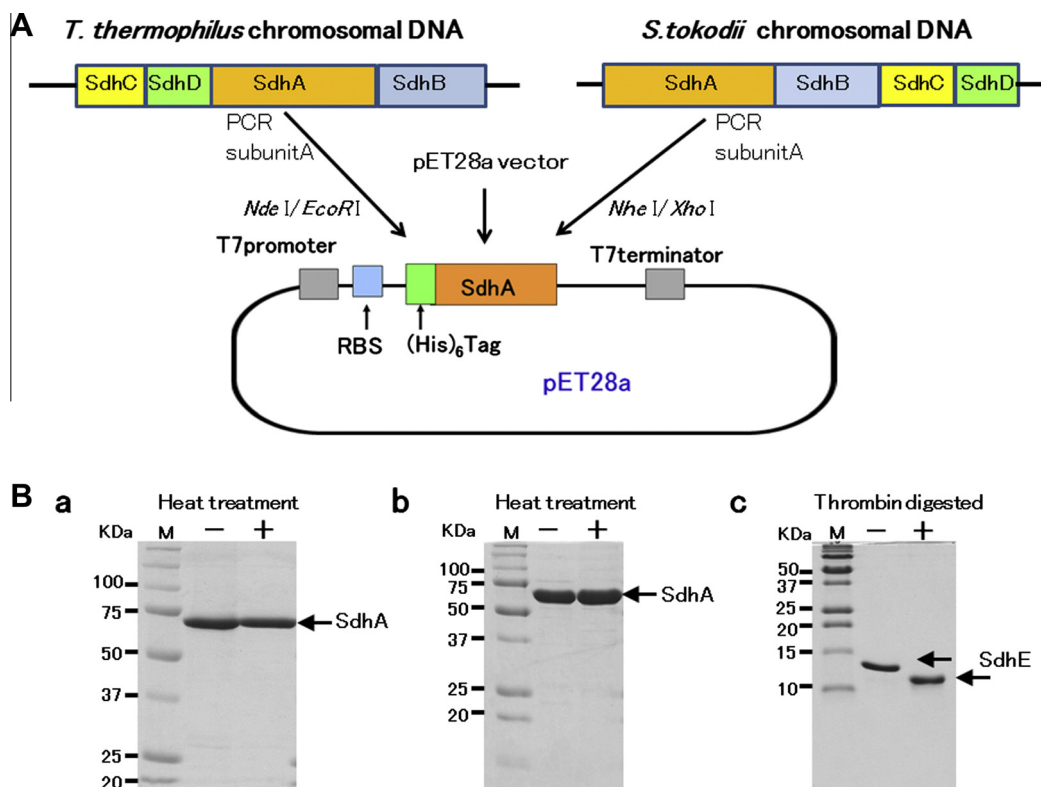

Fig. 1. Strategy for the construction of expression plasmids and their analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. (A) Structures of the expression plasmids TthSdhA–pET28a, StoSdhA–pET28a, and EcoSdhE–pET28a. (B) Recombinant *Thermus thermophilus* succinate dehydrogenase subunit A (SdhA; B-a) and *Sulfolobus tokodaii* SdhA (B-b) were overproduced in *Escherichia coli* without or with heat treatment (B-a: 65 °C, 1 h; B-b: 85 °C, 1 h) in the presence of flavin adenine dinucleotide and succinate. *E. coli* succinate dehydrogenase subunit E was overproduced in *E. coli* without heat treatment and with or without thrombin digestion (B-c). Lane M: molecular weight markers. Proteins were stained using Coomassie brilliant blue R250.

washed extensively with 20 mM potassium phosphate buffer (pH 7.0) containing 300 mM NaCl and 20–50 mM imidazole. The recombinant proteins were eluted with 20 mM potassium phosphate buffer (pH 7.0) containing 300 mM NaCl and 250 mM imidazole. The eluted fractions were loaded onto Sephadex® G-75 columns (Amersham Biosciences AB, Uppsala, Sweden) equilibrated with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.0) containing 250 mM NaCl, and then glycerol was added to a final concentration of 20%. All chemicals used were of analytical grade. Hexahistidine-tagged EcoSdhE was digested in thrombin and collected after passage through a nickel-nitrilotriacetic acid Superflow column at 4 °C.

The content of purified SdhA and SdhE was quantified using the Pierce Coomassie Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA), with bovine serum albumin as a standard.

Polyacrylamide gel electrophoresis (PAGE) was performed on 12% or 15% sodium dodecyl sulfate (SDS) gels after treating the proteins with 2% SDS in the presence of 2% 2-mercaptoethanol at 90 °C for 15 min. The proteins were visualized using Coomassie brilliant blue R250 staining.

2.4. Covalent flavinylation assay and analysis

The reaction solution is 20 mM HEPES buffer (pH 7.0) containing 250 mM NaCl and the following dicarboxylic acid is contained in 20 mM of each sort: succinic acid (Wako Pure Chemical Industries, Ltd., Osaka, Japan), sodium fumarate (Nacalai Tesque, Inc., Kyoto, Japan), sodium malonate (Wako Pure Chemical Industries, Ltd.), and oxaloacetic acid (Wako Pure Chemical Industries, Ltd.). Purified recombinant SdhA (not heat treated) was added to these reaction solutions, and the flavinylation reaction was initiated with 20 µM FAD at the required temperature. If aggregation occurred, the sample was pelleted by 10-min centrifugation (15 000 rpm) at 4 °C, and subsequently all unbound FAD was removed using a nickel-nitrilotriacetic acid Superflow column (Qiagen GmbH) at 4 °C. The column was washed extensively with 20 mM potassium phosphate buffer (pH 7.0) containing 300 mM NaCl and 20–50 mM imidazole. The recombinant proteins were eluted with 20 mM potassium phosphate buffer (pH 7.0) containing 300 mM NaCl and 250 mM imidazole. Absorption spectral analysis of the supernatant was performed using a Shimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) immediately.

The covalent nature of FAD linkage was verified by protein precipitation, which showed whether the yellow flavin was partitioned into the pellet or the supernatant. In brief, recombinant SdhA was mixed with trichloroacetic acid (TCA; Wako Pure Chemical Industries, Ltd.; final 10%) on ice for 30 min. The preparation was centrifuged at 15 000 rpm for 5 min at 4 °C, and the pellet was photographed to determine the color. Quantitation of FAD was performed as described by Blaut et al. [19].

3. Results and discussion

3.1. Heterologous overexpression and characterization of succinate dehydrogenase subunit A of Thermus thermophilus strain HB8 and Sulfolobus tokodaii

Recombinant TthSdhA, StoSdhA, and EcoSdhE with hexahistidine tags at the N terminus were heterologously overproduced in *E. coli* BL21-CodonPlus (DE3)-RIL cells. The 577 – (TthSdhA), 566 – (StoSdhA), and 88 – (EcoSdhE) amino acid products were water soluble. SDS–PAGE showed a single 64-kDa band for ThSdhA, 63-kDa band for StoSdhA, and 10-kDa band for EcoSdhE (Fig. 1B). The amount of purified protein obtained from 4 to 6 g (wet) of *E. coli* cells and 1 L of culture under the applied conditions was approximately 35–50 mg (TthSdhA) and 7–10 mg (StoSdhA), respectively. The initial FAD content purified SdhA was not detected (data not shown).

3.2. Covalent flavinylation of Thermus thermophilus and Sulfolobus tokodaii succinate dehydrogenase subunit A requires heat

The ratio of covalent to non-covalent flavinylation of the recombinant TthSdhA and StoSdhA proteins gradually increased with elevating temperature (Fig. 2 top). The near-ultraviolet/visible absorption spectra of purified TthSdhA (25–65 °C heat-treated) and StoSdhA (85 °C heat-treated) showed absorption peaks at 450 and 356 nm, respectively, characteristic of FAD covalently bound to SdhA (Fig. 2 middle). However, TthSdhA (5–15 °C heat-treated) and StoSdhA (25–75 °C heat-treated) showed absorption peaks at 450 and 374–378 nm, respectively, characteristic of free FAD or FAD non-covalently bound to SdhA (Fig. 2 middle). The TCA precipitates of TthSdhA (15–65 °C heat-treated) and StoSdhA (65–85 °C heat-treated) had a yellow color, typical of flavoproteins containing covalently linked FAD (Fig. 2 bottom). Conversely, the TCA precipitates of TthSdhA (5 °C) and StoSdhA (25–55 °C) were white, and the supernatant was yellow as a result of free FAD. These data indicate that heat is essential for the covalent binding of FAD to both TthSdhA and StoSdhA. TthSdhA recombinant protein aggregated at temperatures exceeding 75 °C (data not shown); the optimum temperature was 65 °C.

Even among thermophilic prokaryotes, the temperature required for the covalent flavinylation of SdhA varies: in this study, it was higher in *S. tokodaii* than in *T. thermophilus*. *T. thermophilus* is a eubacterium; this suggests that this bacterium is in the process of evolving to adapt to lower temperatures. Conversely, *S. tokodaii* is an archaea and exhibits primitive characteristics.

When recombinant TthSdhA and StoSdhA were heat treated for 1–3 h at 15/25 °C or 65/75 °C, the percentage of covalent flavinylation gradually increased (Supplementary Fig. 1A/B). I quantified covalent versus non-covalent FAD binding to compare the coupling efficiency of the flavinylation reaction supported by each sample (Supplementary Table 2). Coupling efficiency was 6–8.3 for the covalent binding of FAD to TthSdhA and 10–11.9 for the covalent binding of FAD to StoSdhA. If 100% of FAD covalent/non-covalent binds to SdhA protein, the value is 15–16 nmol/mg. The 50% of TthSdhA bound FAD and the 70% of StoSdhA bound FAD.

Moreover, in a comparison of the quantification of the covalent binding of FAD to TthSdhA and StoSdhA, coupling efficiency was higher with StoSdhA. Overall, these results indicate a high consistency in coupling efficiency for the covalent binding of FAD to TthSdhA and StoSdhA.

3.3. Covalent flavinylation of Thermus thermophilus and Sulfolobus tokodaii succinate dehydrogenase subunit A requires dicarboxylic acids

Purified TthSdhA and StoSdhA were examined with succinate, fumarate, malonate, and oxaloacetate to identify which supports the covalent flavinylation of SdhA. Oxaloacetate is an inhibitor of *E. coli* FRD [20–22]. Surprisingly, with all dicarboxylic acids, over 70% of FAD linked to TthSdhA was covalently linked (Fig. 3) and the precipitates were yellow (data not shown). Conversely, StoSdhA covalently bound FAD in the presence of succinate and fumarate; less than 20% of FAD bound to StoSdhA was covalently bound in the presence of malate, and the levels of FAD covalently bound to StoSdhA in the presence of oxaloacetate were undetectable. The ratio of covalently to non-covalently bound FAD decreased dramatically when the recombinant proteins were incubated without dicarboxylic acids (data not shown).
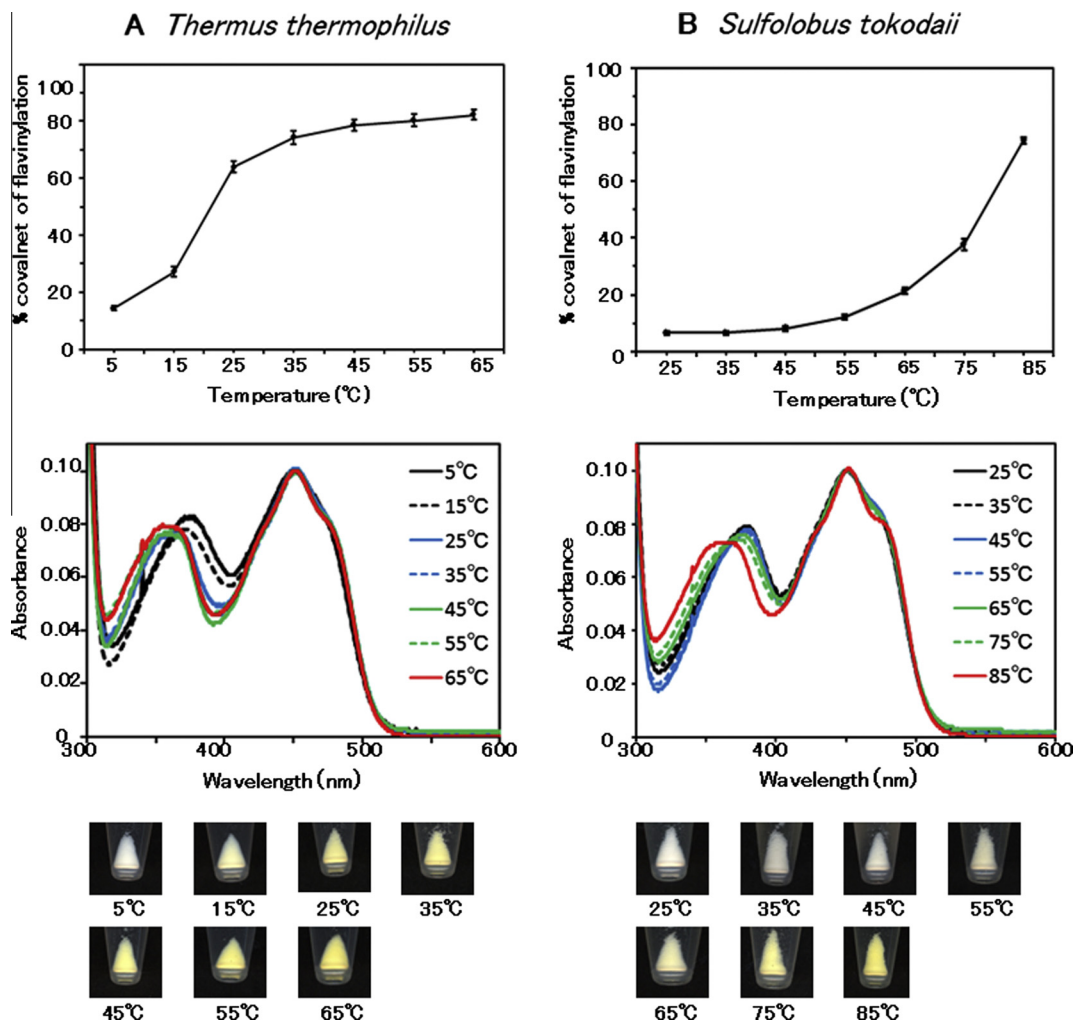
Fig. 2. Temperature dependence of the covalent flavinylation of recombinant succinate dehydrogenase subunit A. (A) *Thermus thermophilus* succinate dehydrogenase subunit A (TthSdhA). (B) *Sulfolobus tokodaii* SdhA (StoSdhA). Top, the percentage of covalent flavinylation; middle, the near-ultraviolet/visible absorption spectra; bottom, the trichloroacetic acid precipitation. All experiments were conducted in the presence of 20 mM succinate and 20 μM flavin adenine dinucleotide. Data are expressed as means ± standard error (n = 4–10); $P < 0.05$.
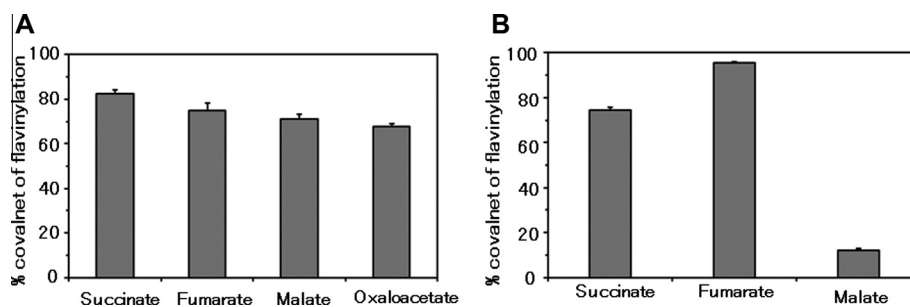

Fig. 3. The dicarboxylic acid requirement of the covalent flavinylation of recombinant succinate dehydrogenase subunit A. (A) *Thermus thermophilus* succinate dehydrogenase subunit A (TthSdhA). (B) *Sulfolobus tokodaii* SdhA (StoSdhA). Samples of TthSdhA were heat treated at 65 °C and StoSdhA at 85 °C for 1 h in the presence of 20 μM flavin adenine dinucleotide. Data are expressed as means ± standard error (n = 5–7); $P < 0.05$. The reaction solution is 20 mM HEPES buffer (pH 7.0) containing 250 mM NaCl and the following dicarboxylic acid is contained in 20 mM of each sort: succinic acid, sodium fumarate, sodium malonate, and oxaloacetic acid.

These data suggest that specific dicarboxylic acids stabilize the structure of SDH in a configuration that facilitates FAD insertion and covalent linkage. Because some of the dicarboxylic acids that stimulate covalent FAD incorporation in TtSdhA and StSdhA do not reduce the flavin cofactor, the involvement of flavin reduction [11] in the autocatalytic covalent flavinylation mechanism remains enigmatic.

3.4. Influence of SdhE on the covalent flavinylation of TthSdhA and StoSdhA

SdhE is considered necessary for covalent flavinylation in eubacteria; thus, I investigated the influence of SdhE on the covalent flavinylation of TthSdhA and StoSdhA. These experiments were conducted at 25 or 35 °C because SdhE was derived from the
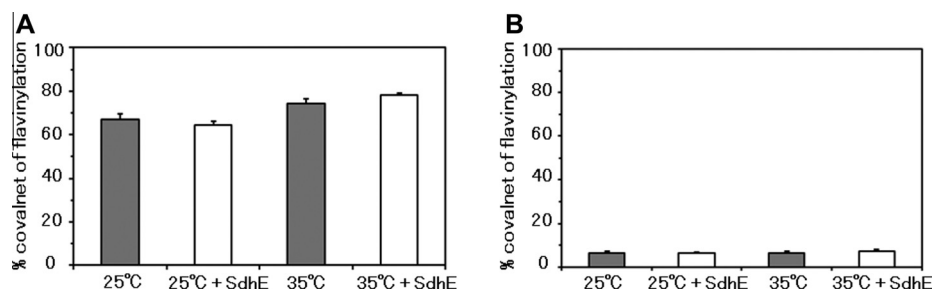
Fig. 4. The effect of succinate dehydrogenase protein E on the covalent flavinylation of recombinant succinate dehydrogenase subunit A. (A) *Thermus thermophilus* succinate dehydrogenase subunit A (TthSdhA). (B) *Sulfolobus tokodaii* SdhA (StoSdhA). All experiments were conducted in the presence of 20 mM succinate and 20 μM flavin adenine dinucleotide. SdhE was added so that the molar ratio would be 1 : 1 in relation to SdhA. Data are expressed as means ± standard error ($n = 5$–7); $P < 0.05$.

mesophilic bacterium *E. coli*. Therefore, the covalent flavinylation of TthSdhA and StoSdhA was unaffected by SdhE (Fig. 4).

The SdhE protein family evolved in α-proteobacteria [18]; bacteria evolutionarily older than ancestral α-proteobacteria lack SdhE. In general, hyperthermophilic archaea and bacteria are located at the roots of the phylogenetic tree. *T. thermophilus* and *S. tokodaii* lack the chaperone-like protein SdhE, and it is supposed that covalent flavinylation relies on heat in the environment and the presence of dicarboxylic acids *in vivo*. Mesophilic bacteria and hyperthermophilic bacteria/archaea exist in environments with different temperatures: mesophilic bacteria exist at temperatures of 35–40 °C and require the chaperone-like protein SdhE for covalent flavinylation, whereas hyperthermophilic bacteria/archaea use thermal energy for covalent flavinylation. More detailed thermodynamic analysis will be necessary in the future.

4. Conclusion

The amino acid sequences of SdhA/FrdA in mesophilic bacteria (*E. coli*) and hyperthermophilic prokaryotes (*T. thermophilus* and *S. tokodaii*) have a high degree of homology (Supplementary Fig. 2). Despite this, SdhA in *E. coli* requires SdhE for covalent flavinylation, whereas SdhA in *T. thermophilus* and *S. tokodaii* requires heat and the presence of dicarboxylic acids instead of SdhE. The results of this study reflect the environmental changes that have occurred on earth over time. Hyperthermophilic bacteria/archaea use thermal energy for covalent flavinylation because they exist in high-temperature environments. As the temperature of the earth gradually decreased, mesophilic bacteria evolved chaperone-like proteins, such as SdhE, to assist in covalent flavinylation. It is interesting to speculate that changes in the global environment and the evolution of SdhE may be related.

Further investigation on mesophilic bacteria lacking SdhE and psychrophilic bacteria, and on other flavoproteins that undergo covalent flavinylation, will be necessary in the future. However, all key residues involved in the proposed catalytic mechanism for succinate oxidation and reverse fumarate reduction in the dicarboxylate binding site of archaeal SdhA are conserved [4,23–25]. In addition, the crystal structures of FRD and SDH have been published [26–29]. Recombinant SdhA protein produced by *T. thermophilus* and *S. tokodaii* is thermostable and the yield is large; it could be used in the site-directed mutagenesis of FAD-SdhA and its catalytic sites.

Acknowledgment

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.02.022.

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