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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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ABSTRACT

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.

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.

Abbreviations: FAD, flavin adenine dinucleotide; FRD, fumarate reductase; SDH, succinate dehydrogenase; SdhA, SDH subunit A; TCA, trichloroacetic acid; LB, lysogeny broth; PCR, polymerase chain reaction; rpm, revolutions per minute; bp, base pairs; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; SdhE, succinate dehydrogenase protein E

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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; 30000 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

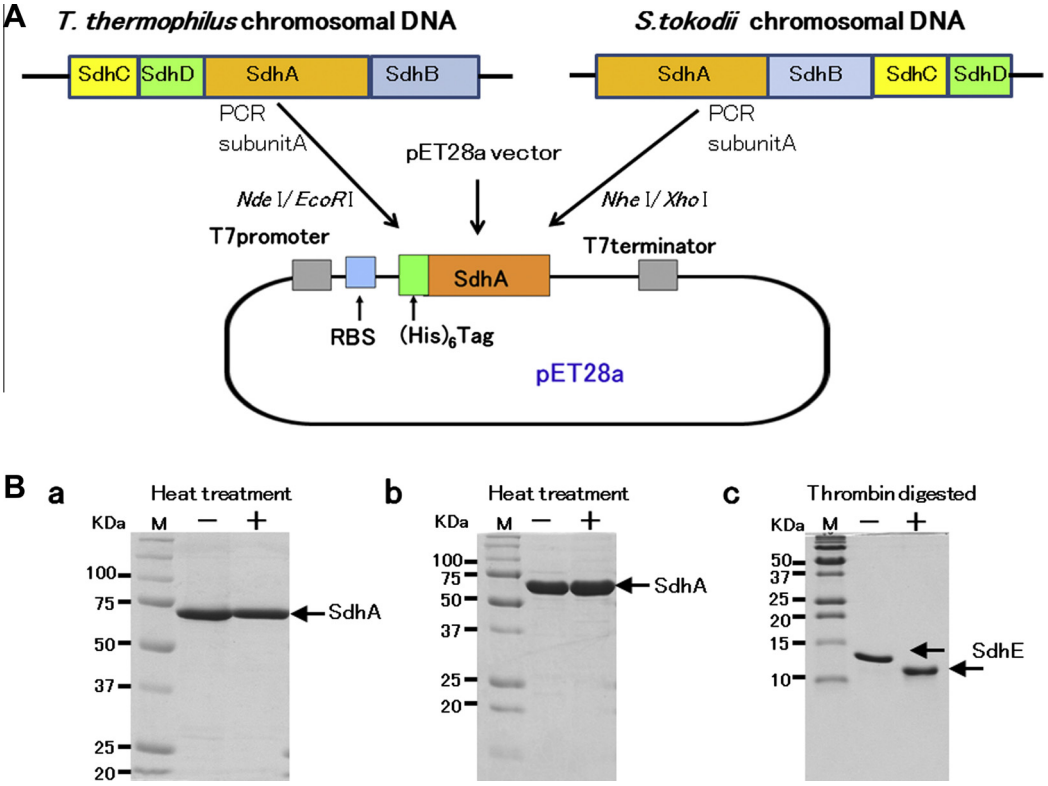

Fig. 1. Strategy for the construction of expression plasmids and their analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. (A) Structures of the expression plasmids TthSdhA–pET28a, StoSdhA–pET28a, and EcoSdhE–pET28a. (B) Recombinant *Thermus thermophilus* succinate dehydrogenase subunit 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.