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In-vitro, SDH5-dependent flavinylation of immobilized human respiratory complex II flavoprotein

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ABSTRACT

Mitochondrial Complex II (Succinate: ubiquinone oxidoreductase) has a covalently bound FAD cofactor in its largest subunit (SDHA), which accepts electrons from oxidation of succinate during catalysis. The mechanism of flavin attachment, and factors involved, have not been fully elucidated. The recent report of an assembly factor SDH5 (SDHAF2, SDHE) required for flavinylation (Hao et al., 2009 Science 325, 1139–1142) raises the prospect of achieving flavinylation in a completely defined system, which would facilitate elucidation of the precise role played by SDH5 and other factors. At this time that goal has not been achieved, and the actual function of SDH5 is still unknown.

We have developed a procedure for in-vitro flavinylation of recombinant human apo-SDHA, immobilized on Ni-IMAC resin by a His tag, in a chemically defined medium. In this system flavinylation has a pH optimum of 6.5 and is completely dependent on added SDH5. The results suggest that FAD interacts noncovalently with SDHA in the absence of SDH5. This system will be useful in understanding the process of flavinylation of SDHA and the role of SDH5 in this process.

1. Introduction

Respiratory Complex II is a membrane protein complex responsible for succinate: ubiquinone reductase activity (SQR; E.C. 1.3.5.1). Situated in the mitochondrial inner membrane, it catalyzes the TCA cycle reaction, oxidation of succinate to fumarate, in the matrix. The reducing equivalents from that reaction are used to reduce ubiquinone to ubiquinol in the membrane. It consists of four subunits denoted SDHA, B, C, and D (SDH1p, 2p, 3p, and 4p in yeast). Crystal structures are available for complex II of *Escherichia coli* [1–3], pig [4,5], chicken [6,7], and *Ascaris suum* [8–10].

The largest subunit, SDHA, contains the succinate oxidation site adjacent to a FAD moiety assumed to be the initial acceptor of electrons from succinate. The FAD is covalently bound via an 8α-(N3-histidyl)-linkage [11] to His56 (mature bovine sequence) [12]. This covalent linkage is not required for FAD incorporation or for assembly of the complex, either in yeast Complex II [13] or with the related *E. coli* fumarate reductase (FRD) [14]. However the modification of the isoalloxazine ring by covalent histidyl linkage raises the midpoint potential of the flavin allowing oxidation of succinate [15]. Without this modification the enzyme does not oxidize succinate at an appreciable rate [13,14].

Brandsch and Bichler [16] studied flavinylation of SQR and FRD in *E. coli*. They over-expressed the complexes from plasmids containing the operons, in flavin-deficient mutants. After lysing the cells, radioactive FAD was added along with other components, and incorporation of flavin in SDHA was measured. It was found that the TCA cycle intermediates citrate, isocitrate, succinate, and fumarate stimulated flavinylation in this system.

In eukaryotes SDHA is synthesized with a leader sequence which directs its import into the mitochondria. Robinson and Lemire [17,18] investigated the requirements for flavinylation of apo-SDHA in yeast. When in-vitro translated pre-protein was incubated with mitochondria it was imported into the mitochondria, the leader sequence was cleaved, and the protein became flavinated [17]. Cleavage of the presequence is required for flavinylation but the import step is not, as pre-protein added to a mitochondrial lysate (matrix preparation) was cleaved and flavinated [18].

Abbreviations are used: SDH, succinate dehydrogenase (Complex II); IPTG, isopropyl β-D-1-thiogalactopyranoside; 2-ME, 2-mercaptoethanol; SDS-PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate; IMAC, immobilized metal affinity chromatography; Ni-IDA, nickel iminodiacetic acid; RCF, relative centrifugal force; RFI, relative fluorescence intensity; OD600, optical absorbance at 600 nm.

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Dicarboxylates (succinate, fumarate, malate) stimulated but were not required. ATP also stimulated flavinylation, and ATP depletion or treatment of the matrix fraction with protease or N-ethylmaleimide inhibited [18]. Some unknown factor(s) in the matrix preparation were required. Later it was discovered [19] that a paralog of HSP-60, named TCM62, was required for maturation/flavinylation in vivo. This protein, which has no known homolog in mammals, was later proposed to have a more general role in protecting mitochondrial proteins under conditions of heat stress [20].

Recently a mitochondrial protein of unknown function was found to be required for flavinylation of SDHA in humans and in yeast, and was renamed SDH5 [21] or SDH Assembly Factor 2 (SDHAF2). A mutation in this protein was found to correlate with occurrence of paraganglioma in a familial line of this disease. The human protein with the disease mutation, expressed in yeast or human cells, failed to support flavinylation of SDHA in cells lacking endogenous SDH5. Similar proteins were found to be required for flavinylation of SDHA in enterobacteria [22] and for assembly and activity of Complex II in *Arabidopsis* [23].

These authors [21] also showed that yeast SDH1p expressed in *E. coli* was flavinated within the bacterial cell if, and only if, yeast SDH5 was co-expressed. It was concluded that the role of SDH5 could be in catalyzing the flavinylation reaction, in keeping SDHA in a state competent to be flavinated (chaperone role), or in delivering FAD to SDHA [21]. Subsequent experiments have not differentiated between these non-mutually-exclusive possibilities, although NMR studies failed to show any interaction between purified yeast SDH5 and flavin [24], and evidence has been obtained for binding of SDH5 to apo-SDHA [21,25,26], making a flavin delivery role less likely and a chaperone or catalytic role more likely.

These results suggest that SDH5 may have been the crucial ingredient in the matrix preparation of Robinson and Lemire's in-vitro flavinylation, in which case it might be possible now to demonstrate in-vitro flavinylation in a completely defined system using recombinant proteins, pure FAD and other small molecules. This would allow further elucidation of the role of SDH5 and determination of the factors required and the order in which they are required. This seemingly simple experiment has not been reported in the literature, and our preliminary attempts were unsuccessful, suggesting that other factors may be involved. Through the experiments described here we have obtained a better understanding of the requirements, and we have developed a defined system for studying the SDH5-dependent flavinylation reaction in vitro.

2. Experimental procedures

2.1. Engineered expression constructs

The coding sequences for human SDHA (MGC-1484) and SDH5 (SDHAF2, MGC-4724) from the Mammalian Gene Collection were obtained from American Type Culture Collection. The coding sequences were amplified from the cloning vectors and inserted into expression vectors as follows.

The expression vector for N-terminally His_6-tagged mature human SDHA (H_6hSDHA) was constructed by placing the nucleotides coding for the mature sequence (starting with residues 42ASAK, through the stop codon) into the first multicloning site of the pETDuet™-1 vector between the *Bam*HI and *Eco*RI restriction sites. This results in prepending 14 residues MGSSH_6SQDP from coding sequence in the vector.

For expression of hSDH5 in *E. coli*, we designed a putative mature sequence starting with 39SPTD. Primers were designed to amplify this sequence with extensions to insert it between the *Nde*I and *Xho*I restriction sites of the pET41 vector. The bases of the *Nde*I site provide the initiator Met while the *Xho*I site and following bases of the vector append LEH_8 on the C terminus.

A second construct, in which nucleotides coding for the His_8 tag were deleted but the linker residues LE encoded by the restriction site were retained, was designed for expression of tag-less mature hSDH5 for pulldown studies with H_6hSDHA. and conventional purification. If not otherwise stated, it was this tag-less, mature construct **hSDH5**, that was used in the flavinylation experiments described here. The predicted sequence of the encoded protein (**hSDH5**) is:

MSPTDSQKDMIEIPLPPWQERTDESIETKRARLLYESRKRGMLENCILLSLFAKEHLQHMTEKQLNLY
DRLINEPSNDWDIYYWATEAKPAPEIFENEVMALLRDFAKNKNKEQRLRAPDLEYLFEKPRLE

with a molecular weight of 15,756 (15,625 if the initial Met is removed).

It has since been reported that mature hSDH5 starts with 37**GD**SPDT… [25]. Thus our construct starting with residue Ser39 may lack the N-terminal **GD** residues of the actual mature protein, or have them replaced by the initiator methionine.

Three additional tag-less constructs were made from this with the paraganglioma-related mutation (G78R) or mutation of the single Cys residue (C84D or C84S). For co-expression in *E. coli*, hSDH5 coding sequences were placed in the second cloning site of the above-described pET-duet-1 H_6hSDHA expression vector, between *Nde*I and *Xho*I restriction sites, with or without a C-terminal His_5 tag with no linker.

Expression Plasmids for H_6hSDHA, hSDH5, and three mutant hSDH5 are being made available through the Addgene plasmid deposition service.

2.2. Procedures

Protein determination was by the Lowry assay [27] modified by inclusion of 1% SDS in the alkaline reagent A [28]. Relative centrifugal force (RCF) is reported at $\mathbf{r_{ave}}$ or as indicated by the instrument.

2.3. Cell culture

E. coli BL21 cells transfected with the various plasmids as well as pRARE plasmid (From Novagen Rosseta2 cells) were grown in rich LB (10 g NaCl, 10 g Tryptone, 5 g yeast extract and 2 g glucose in 1 L of distilled water) at 30 °C. Antibiotics were included to maintain the plasmids: Ampicillin for pETDuet, Kanamycin for pET41, and chloramphenicol for pRARE. The cultures were grown to an OD_{600} of 0.6 before induction with 0.2 mM of IPTG. About 4 h after induction they were harvested by centrifugation. For preparing cell lysate for protein purification and in vitro flavinylation, cells were frozen at −80 °C to accumulate multiple harvests which were combined and processed together as described below.

2.4. Cell lysate preparation

To prepare clarified cell lysate, the cell pellet was resuspended with 2 ml/g wet weight of lysis buffer (0.2 M Tris, pH8.0 and 2 mM