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Over-expression in *Escherichia coli*, purification and characterization of isoform 2 of human FAD synthetase

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Abstract

FAD synthetase (FADS) (EC 2.7.7.2) is a key enzyme in the metabolic pathway that converts riboflavin into the redox cofactor FAD. The human isoform 2 of FADS (hFADS2), which is the product of *FLAD1* gene, was over-expressed in *Escherichia coli* as a T7-tagged protein and identified by MALDI-TOF MS analysis. Its molecular mass, calculated by SDS–PAGE, was approx. 55 kDa. The expressed protein accounted for more than 40% of the total protein extracted from the cell culture; 10% of it was recovered in a soluble and nearly pure form by Triton X-100 treatment of the insoluble cell fraction. hFADS2 possesses FADS activity and has a strict requirement for $MgCl_2$, as demonstrated in a spectrophotometric assay. The purified recombinant isoform 2 showed a kcat of $3.6 \times 10^{-3}\ s^{-1}$ and exhibited a K_M value for FMN of about 0.4 μM. The expression of the hFADS2 isoform opens new perspectives in the structural studies of this enzyme and in the design of antibiotics based on the functional differences between the bacterial and the human enzymes.

Keywords: FAD synthesis; FAD synthetase; FADS; FLAD1; FMN adenylyl transferase; Isoforms

Flavoproteins are a broad class of enzymes, mainly located in mitochondria, that catalyze a variety of biological redox reactions and require the riboflavin-derived redox cofactors, FAD and FMN. In mammals, flavin cofactors are obtained from dietary riboflavin (vitamin B2), delivered to peripheral districts in tight association with serum albumin [see 1–3 for reviews]. The circulating vitamin is efficiently imported into the peripheral cells via specific plasma membrane transporter(s) [3], whose molecular counterparts in higher eukaryotes are unknown. In *Saccharomyces cerevisiae*, *MCH5* has been identified as the first eukaryotic gene encoding a plasma membrane riboflavin transporter [4].

Once inside the cells riboflavin is rapidly converted into catalytically active cofactors via the sequential actions of ATP:riboflavin phosphotransferase (riboflavin kinase (FK)[2] (EC 2.7.1.26)), which converts the vitamin into FMN, and ATP:FMN adenylyltransferase (FAD synthetase (FADS) (EC 2.7.7.2)), which adenylates FMN to FAD. Both enzymes have been purified from *S. cerevisiae* and rat tissues, and biochemically characterized [5–10].

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[2] *Abbreviations used:* BSA, bovine serum albumin; D-AAO, D-amino acid oxidase; D-Ala, D-alanine, ESI-MS/MS, electrospray ionization tandem mass spectrometry; FADS, FAD synthetase; FK, riboflavin kinase; hFADS1, human FAD synthetase isoform 1; hFADS2, human FAD synthetase isoform 2; LB, Luria–Bertani; LDH, lactate dehydrogenase; MALDI-TOF MS, matrix assisted laser desorption time of flight mass spectrometry; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

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In contrast with eukaryotes, most prokaryotes have a single bifunctional protein that provides both FK and FADS activities [11–13]. Monofunctional prokaryotic FKs have been cloned and characterized [14,15].

The first eukaryotic gene encoding a monofunctional FK was identified in *S. cerevisiae* and named *FMN1* [16]. This gene codes for a membrane-bound protein located in both microsomes and the inner mitochondrial membrane. Other FKs have been cloned from *S. cerevisiae* and plants [17,18]. The human ortholog of Fmn1p is an 18.5 kDa protein encoded by the *RFK* gene, whose crystal structure has been recently determined [19].

The first eukaryotic gene for a monofunctional FADS was identified in *S. cerevisiae* and named *FAD1* [20]. It encodes a 35 kDa soluble cytosolic enzyme. In contrast to Fmn1p, which is orthologous to the bacterial bifunctional enzymes, Fad1p belongs to the PAPS-reductase family and has little or no sequence similarity to the bacterial enzymes, such as *ribF* in *E. coli*. Since the latter is required for *E. coli* viability and is unrelated to mammalian enzymes, FADS is a potential target for the development of novel antimicrobial drugs [12].

The subcellular localisation of FADS in eukaryotes is a matter of debate. De Luca and Kaplan first demonstrated that the enzyme responsible for FAD formation is located in the cytosol of rat liver [21]. Since then, FADS has been purified from rat liver cytosolic fractions only as a protein with Mr = 53 kDa in SDS–PAGE [7,10]. Thus, for many years it was assumed that mammalian FAD biosynthesis occurred only in the cytosol. However, using cell fractionation and activity measurements, we have demonstrated the presence of FADS activity in mitochondria from rat liver [22,23] and *S. cerevisiae* [24,25].

The hypothesis that different isoforms with compartment-specific functions may exist in eukaryotes has been recently supported, in humans, by the cloning and functional characterisation of two products of *FLAD1* gene, the putative human ortholog of *FAD1* [26].

Analysis of FADS isoform 1 (hFADS1) and isoform 2 (hFADS2) sequences by iPSORT, TargetP and MITOPROT protein sorting signal prediction programs indicated that hFADS1 has an N-terminal mitochondrial targeting peptide, whereas hFADS2 has not. Therefore, hFADS1 could represent the mitochondrial FADS isoenzyme [26].

In this paper we report the cloning, over-expression and functional characterization of hFADS2, which is the product of *FLAD1* transcript variant 2 (GenBank Accession No. NM201398).

Materials and methods

Materials

All chemicals were from Sigma–Aldrich, if not otherwise specified. *E. coli* Rosetta(DE3) strain and pET-21a(+) plasmid were purchased from Novagen. Restriction endonucleases and other cloning reagents were purchased from Fermentas. Bacto peptone and bacto yeast extract were from Difco.

Cloning of cDNA coding for hFADS2

The 1473 bp cDNA corresponding to the encoding sequence for hFADS2 (UniProtKB/TrEMBL Accession No. Q8WU93; GenPept Accession No. NP_958800) was amplified from the full length cDNA RZPD (IMAGp998D2411612) clone (inserted in *Eco*RV/*Not*I sites of pCMV-SPORT6 vector) with the forward and reverse primers 5′-AGCCGAATTCATGACATCTAGGGCC-3′ and 5′-GACCCTCGAGTCATGTGCGGGAGTT-3′, containing the *Eco*RI and *Xho*I sites, respectively. The amplified cDNA was then cloned in the *Eco*RI/*Xho*I sites of the pET-21a(+) expression vector. The resulting recombinant plasmid, defined as pET-21a(+)-hFADS2, encodes a T7-tagged fusion protein corresponding to hFADS2 carrying the extra N-terminal sequence MASMTGGQQMGRG SEF.

Expression of recombinant hFADS2 protein in E. coli

To obtain the recombinant hFADS2 protein, the expression host *E. coli* Rosetta(DE3) strain was transformed with the pET-21a(+)-hFADS2 plasmid by calcium chloride treatment. Selection of transformed colonies was performed on LB-agar plates containing 100 μg/mL ampicillin and 34 μg/mL chloramphenicol. *E. coli* Rosetta(DE3) cells carrying the recombinant plasmid were inoculated in 100 mL of LB medium (1% bacto peptone, 0.5% bacto yeast extract, 1% NaCl, pH 7.0) supplemented with 100 μg/mL ampicillin and 34 μg/mL chloramphenicol, and cultured overnight at 30 °C with rotary shaking (≅200 rpm). A 50-mL aliquot of the cell culture was transferred to 0.5 L of fresh LB medium supplemented with 100 μg/mL ampicillin and 34 μg/mL chloramphenicol and 0.4 mM IPTG, to induce the expression of the recombinant protein. Growth was continued for 1–4 h at 30 °C, then bacteria were harvested by centrifugation at 3000*g* for 10 min at 4 °C and the pellet stored at −20 °C. The bacterial pellet (about 3 g wet weight) was thawed on ice for 15 min and resuspended in 50 mL of 1 × T7-Tag bind/wash buffer (4.29 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl, 0.1% Tween 20, and 0.002% sodium azide, pH 7.3) supplemented with 0.2 mL of protease inhibitor cocktail (P8849, Sigma–Aldrich) and 0.5 mM PMSF. Cells were disrupted by mild sonication at 4 °C (three pulses for 60 s, 30 s and 30 s at 70–100 W with 60 s intermission) using a branson sonifier 250. The soluble and the insoluble cell fractions were separated by centrifugation of the cell lysate at 20,000*g* for 30 min at 4 °C. The supernatant, containing the soluble over-expressed protein, was used for the analysis of FADS activity.

Purification of functional recombinant hFADS2

The insoluble fraction obtained as described above, was washed twice with 50 mM Tris/HCl, pH 7.5, and centrifuged at 20,000*g* for 10 min at 4 °C. The resulting pellet was treated with 3% Triton X-100 and centrifuged at 20,000*g*