# Functional Expression of Human Mitochondrial CYP11B2 in Fission Yeast and Identification of a New Internal Electron Transfer Protein, etp1[†]

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ABSTRACT: Mitochondrial cytochrome P450 enzymes play a crucial role in the steroid biosynthesis in human adrenals, catalyzing regio- and stereospecific hydroxylations. In search of a new model system for the study of these enzymes, we expressed the human CYP11B2 (aldosterone synthase, $P450_{aldo}$) in fission yeast *Schizosaccharomyces pombe*. Analysis of the subcellular localization of the P450 enzyme by Western blot analysis, fluorescence microscopy, and electron microscopy demonstrated that the mitochondrial localization signal of the human protein is functional in *S. pombe*. The transformed yeasts show the inducible ability to convert in vivo considerable amounts of 11-deoxycortisol to cortisol and 11-deoxycorticosterone to corticosterone, 18-hydroxycorticosterone, and aldosterone, respectively. Although in mammalian cells, mitochondrial steroid hydroxylases depend for their activity on an electron transport chain that consists of two proteins, adrenodoxin and adrenodoxin reductase, no coexpression of these proteins is needed for efficient substrate conversion by intact fission yeast cells. Searching the fission yeast genome for adrenodoxin homologues, a gene was identified that codes for a protein with an amino terminal domain homologous to COX15 of *Saccharomyces cerevisiae* and a carboxy terminal ferredoxin domain. It was found that overexpression of this gene significantly enhances steroid hydroxylase activity of CYP11B2 expressing fission yeast cells. Moreover, the bacterially expressed ferredoxin domain of this protein can replace adrenodoxin in a reconstituted steroid hydroxylation assay and transfer electrons from adrenodoxin reductase to a mammalian or a bacterial cytochrome P450. Therefore, we suggest to name this protein etp1 (electron-transfer protein 1).

Mitochondrial cytochrome P450 enzymes play a crucial role in steroid biosynthesis in human adrenals, catalyzing regio- and stereospecific hydroxylations (*1*−*4*). The initial step in steroid biosynthesis, the side-chain cleavage reaction performed on cholesterol yielding pregnenolone, is performed by CYP11A1, while two other P450s, CYP11B1 and CYP11B2, catalyze the last steps of cortisol and aldosterone biosynthesis, respectively. These three P450 enzymes obtain the electrons necessary for the oxygen activation and steroid hydroxylation from a NADPH-dependent redox system consisting of the flavoprotein AdR[1] and the iron−sulfur protein Adx. Adx belongs to the family of [2Fe-2S] ferredoxins which are generally low molecular weight proteins that are widely distributed among bacteria, plants, and animals and participate in a variety of electron-transfer reactions (*5*, *6*); it is a soluble protein located in the matrix of adrenal mitochondria and has been isolated from different mammalian species.

In general, mitochondrial cytochromes P450 are more difficult to express in microorganisms than their microsomal relatives. While some mitochondrial P450s such as bovine CYP11A1 (*7*), rat CYP24 (*8*), and CYP27 (*9*) could be readily expressed in *Escherichia coli*, expression of CYP11B1 and CYP11B2 from rat was achieved with much lower yield (*10*). Expression of a modified bovine CYP11B1 whose natural presequence was replaced by a yeast mitochondrial localization signal in *Saccharomyces cerevisiae* was demonstrated, but the functional expression of a human CYP11B enzyme in a unicellular organism has not been reported so far. Therefore, activity studies on these enzymes were limited to stable (*11*) or transient (*12*, *13*) transfection experiments with mammalian cell lines.

The use of fission yeast as a host for the expression of a microsomal cytochrome P450, CYP2C11, was first reported by Yamazaki et al. who also demonstrated that *Schizosaccharomyces pombe* has a very low level of both endogenous P450 and NADPH−P450 reductase (*14*). Therefore, this group measured steroid hydroxylation activity not in living cells but in membrane fractions that were supplied with an

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[1] Abbreviations: Adx, adrenodoxin; etp1, electron-transfer protein 1; AdR, adrenodoxin reductase; COX, cytochrome *c* oxidase; $etp1^{cd}$, COX15 homology domain of etp1; $etp1^{fd}$, ferredoxin domain of etp1; DOC, 11-deoxycorticosterone; RSS, 11-deoxycortisol; MPP, matrix processing peptidase; MIP, mitochondrial intermediate peptidase.

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Table 1: Fission Yeast Strains Used in This Study

| strain | genotype | ref |
|---|---|---|
| NCYC2036 | h⁻ ura4.dl18 | *57* |
| MB164 | *NCYC2036/pINT5-CYP11B2 integrant* | this study |
| 1445 | h⁻ ade6.M210 leu1.32 ura4.dl18 his3.Δ1 | *58* |
| MB192 | *1445/pREP41−CYP11B2 Pk N* | this study |
| MB193 | *1445/pREP41−CYP11B2 Pk C* | this study |
| MB224 | *1445/pREP41−CYP11B2 Pk C/ pREP42-etp1 Pk C* | this study |
| MB241 | *1445/pREP41-EGFP C* | this study |
| MB249 | *1445/pREP41−CYP11B2 EGFP C* | this study |

excess amount of rabbit reductase. Later, in a direct comparison of *S. pombe* with *S. cerevisiae* expressing human CYP2C9 it was found that both specific P450 content and in vitro hydroxylase activity were approximately 10-fold higher in fission yeast microsomes as compared to those of bakers yeast (*15*). Moreover, the yield of CYP2C9 holoenzyme produced in fission yeast amounted to about 10% of total cellular protein content. Because of these encouraging results, we suspected that fission yeast might also be a suitable host for the expression of mitochondrial cytochromes P450.

We expressed human CYP11B2 in *S. pombe* and investigated its properties. It was found that the unmodified mitochondrial localization signal of this steroid hydroxylase is functional in fission yeast as the enzyme is efficiently targeted to that organelle. This result was unexpected and contrasts to the situation in *S. cerevisiae* where mitochondrial targeting of bovine CYP11B1 depended on the fusion of a COX6 prepeptide to the amino terminus of the enzyme (*16*). Moreover, fission yeast cells expressing human CYP11B2 can in vivo convert DOC to corticosterone, 18-hydroxycorticosterone, and aldosterone and RSS to cortisol, respectively. We demonstrate that this in vivo activity of the mitochondrial P450 enzymes is enhanced after overexpression of the etp1 protein, a fission yeast homologue of mammalian adrenodoxins. Finally, we show that the purified etp1$^{fd}$ can transfer electrons from AdR to a mammalian (CYP11B1) or a bacterial (CYP106A2) cytochrome P450 in an in vitro assay.

## EXPERIMENTAL PROCEDURES

*Chemicals.* Radioactive steroids were obtained from Amersham Pharmacia Biotech (Freiburg, Germany) or NEN (Boston, MA), and nonradioactive steroids were from Sigma (Deisenhofen, Germany).

*Media and General Techniques.* Media and genetic methods for studying fission yeast have been described in detail (*17*, *18*). In addition, we used EMMG (EMM containing 3% glycerol and 0.5% glucose) for respirative growth. General DNA methods were performed using standard techniques (*19*). *S. pombe* strains used in this study are listed in Table 1. Thiamine was used at a concentration of 5 $\mu$M throughout. Cells were generally cultivated at 30 °C and shaking at 170 rpm.

*Expression and Tagging of Human CYP11B2 in Fission Yeast.* For detection of cytochrome P450s by Western blot analysis, fluorescence, and electron microscopy, the CYP11B2 cDNA was cloned as a *Nde*I/*Bam*HI fragment into vectors pREP41−Pk C and pREP41-EGFP C, respectively (*20*). Plasmids were used to transform strain 1445 bearing the *leu1.32* mutation and leucine prototrophs selected. All constructs were verified by automatic sequencing using a LiCor sequencer (MWG-Biotech, Ebersberg, Germany). The sequence of CYP11B2 cloned into pREP41−Pk C differed from the sequence reported by Kawamoto et al., (*21*) at these side-chains: Ser243Asn, Arg249Ser, and Glu258Gly.

*Cloning and Bacterial Expression of etp1$^{fd}$.* The cDNA coding for the carboxy terminal part etp1 was amplified by PCR from genomic fission yeast DNA with primers introducing an artificial ATG start codon instead of Pro-504 and *Xba*I/*Sac*I restriction sites and cloned into pMOSBlue (Amersham) to yield pMOS-etp1$^{fd}$. The etp1$^{fd}$ fragment was excised by *Xba*I/*Sac*I double digest from pMOS-etp1$^{fd}$ and inserted into a modified pTrc99A vector lacking the *Nco*I site and the ATG start codon. *E. coli* strain BL21(DE3) was used as an expression host. Expression was performed at 37 °C in nutrient broth medium for 24 h after induction with IPTG. Cells were harvested and resuspended in 20 mM Tris/Cl buffer, pH 8.5, containing 10 mM EDTA and 20 mM NaCl. After addition of phenylmethylsulfonyl fluoride (50 $\mu$g/mL), cells were sonicated, the suspension was ultracentrifuged, and the etp1$^{fd}$-containing supernatant was used for further purification. After a 2-fold dilution of the supernatant with 20 mM Tris/Cl, pH 7.5, the solution was loaded onto a DEAE-column and eluted with 20 mM Tris/Cl, pH 7.5, containing 500 mM potassium chloride. Etp1$^{fd}$ containing fractions were collected and concentrated for the following size exclusion chromatography. Gel filtration was performed using Sephadex G-50 and 10 mM potassium phosphate, pH 7.4, with a constant flow rate of 0.2 mL/min. The fractions were checked spectrophotometrically for their etp1$^{fd}$ content. Fractions with a Q-value ($A_{415}/A_{276}$) higher than 0.85 were collected, concentrated to a final concentration of 1 mM using centriprep 10 (Amicon; Berverly, MA), and stored at −20 °C until use.

*Functional Replacement of Adx by etp1$^{fd}$ in a Substrate Conversion Assay with CYP11B1 or CYP106A2.* The cytochrome P450-dependent substrate conversion assay was performed in a reconstituted system similar to that described for CYP11A1 by Sugano et al. (*22*). Recombinant bovine Adx and AdR were purified as reported previously (*23*, *24*). Isolation of CYP11B1 from bovine adrenal glands was done according to Akhrem et al. (*25*) with slight modifications, while purification of CYP106A2 from recombinant *E. coli* was done as described by Simgen et al. (*26*). The CYP11B1-dependent conversion of DOC to corticosterone was performed using 0.5 $\mu$M AdR, 0.05 $\mu$M CYP11B1, 40 $\mu$M DOC, between 1 $\mu$M and 64 $\mu$M etp1$^{fd}$ or Adx, and an NADPH regenerating system (containing 5 $\mu$M glucose-6-phosphate, 1 $\mu$M $MgCl_2$, and 1 U glucose-6-phosphate dehydrogenase). The reaction was started by addition of 100 $\mu$M NADPH and incubated for 15 min at 37 °C. After chloroform extraction and drying of the sample, steroids were analyzed by HPLC using a C18 reversed phase column (Waters; Milford, MA) and an isocratic solvent system containing acetonitrile/water (3:2). CYP106A2-dependent conversion of DOC to 15-hydroxy-11-deoxycorticosterone was performed in the same way using CYP106A2 instead of CYP11B1. The results shown were calculated from three independent experiments in all cases.

*Immunochemical Analysis.* The detection of proteins after blotting onto nitrocellulose was performed using a monoclonal anti-Pk tag antibody (MCA1360, Serotec; Oxford,

UK) or polyclonal rabbit antibodies 2439 and 2440 (Biogenes; Berlin, Germany) raised against bacterially expressed etp1,[fd] respectively, peroxidase-conjugated secondary antibodies and 4-chloro-1-naphthol. As a reference for the amount of protein detected by the anti-Pk tag antibody, we used Positope protein (R900-50, Invitrogen; Groningen, Netherlands).

*Protein Sequence Alignments and Phylogenetic Analysis.* Protein sequences were obtained from SWISS−PROT database release 38 (http://www.expasy.ch/sprot/); mitochondrial localization sequences were omitted. Alignments and trees were made using the programs ClustalW (http://pbil.univ-lyon1.fr/), ClustalX (ftp://ftp.ebi.ac.uk/pub/software/dos/clustalw/clustalx/), and PHYLIP Version 3.57c of J. Felsenstein (http://evolution.genetics.washington. edu/phylip.html), applying the NJ (Neighbor Joining) method of Saitou and Nei.

*Microscopy.* Fluorescence microscopic images were obtained with a cooled, charge-coupled device as image detector. A Peltier-cooled camera (Photometrics Ltd; Tucson, AZ) was attached to an Olympus (Hamburg, Germany) inverted microscope IX70; the microscope lamp shutter, focus movement, data collection, and filter combinations were controlled by a Silicon Graphics (Mountain View, CA) workstation. Details of the microscope system setup are described in Haraguchi et al. (*27*). Immunoelectron microscopy was carried out as described previously (*28*, *29*). Briefly, fission yeast cells were harvested by centrifugation and fixed for 1 h with 4% formaldehyde and 0.5% glutaraldehyde under culture conditions (pH 5.5, 30 °C), cryoprotected by a mixture of 25% polyvinylpyrrolindone (PVP K15, molecular weight 10 000; Fluka; Taufkirchen, Germany) and 1.6 M sucrose (*30*) for 3 h, and frozen in liquid nitrogen. Ultrathin cryosections were prepared with glass knifes and transferred to Formvar-carbon-coated copper grids using the cryoprotectant mixture. Primary antibody was monoclonal anti-Pk tag IgG. Labeling with primary antibody and secondary antibody-gold complexes (10 nm, Dianova; Hamburg, Germany) was performed as described elsewhere (*31*). Finally, the sections were stained and stabilized by a freshly prepared mixture of 3% tungstosilicic acid hydrate (Fluka) and 2.5% poly(vinyl alcohol) (molecular weight 10 000; Sigma).

*In Vivo Steroid Hydroxylase Assays.* Exponentially growing fission yeast cells were cultivated by shaking at 30 °C in 10 mL of EMM or EMMG containing supplements as required and steroid substrates at the indicated concentrations. For detection of steroids, 0.6 $\mu$Ci [$^3$H]RSS or 10 nCi [$^{14}$C]-DOC were added to each flask, respectively. After 24 h, 500 $\mu$L of each culture supernatant was extracted twice with chloroform, and the organic phases were combined and dried under vacuum. The residues were dissolved in 10 $\mu$L of chloroform and spotted onto glass-backed silica-coated HPTLC plates (Kieselgel 60 $F_{254}$, Merck; Darmstadt, Germany). In addition, small amounts of nonradioactive steroids were spotted as references. The HPTLC was developed twice in chloroform/methanol/water (300:20:1; v/v), and steroids were identified after exposure to Fuji imaging plates and quantitated on a phosphoimager (BAS-2500, Fuji; Stamford, CT). All data given were calculated from at least two independent experiments and were normalized to equal cell concentrations.

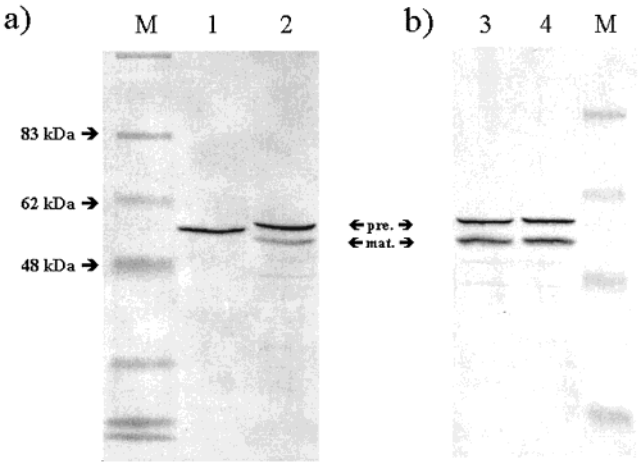


FIGURE 1: Immunological detection of human CYP11B2 expressed in *S. pombe*. (a) Fission yeast strains were grown in the absence of thiamine, and total protein lysates were prepared and analyzed by SDS−PAGE and Western blotting using an α-Pk antibody. Lane 1, MB192 (expressing CYP11B2−Pk N), lane 2, MB193 (expressing CYP11B2−Pk C). (b) Comparison of CYP11B2 expression with or without overexpression of etp1. Fission yeast strains MB193 (CYP11B2−Pk C) and MB224 (CYP11B2−Pk C and etp1-OP) were grown in the absence of thiamine, and total protein lysates were prepared and analyzed as above. pre, preprotein; mat, mature protein.

*In Vitro Translation of etp1*. Full-length etp1 was cloned into pcDNA3.1/CT-GFP-TOPO (Invitrogen) to yield plasmid pMB131; for in vitro translation TNT T7 kit (Promega; Mannheim, Germany) and [$^{35}$S]methionine (Amersham) were used, and the reaction was conducted as recommended by the supplier. Reaction products were separated by SDS−PAGE, blotted onto nitrocellulose, and identified after exposure to Fuji imaging plates as above.

## RESULTS

*Expression of Human CYP11B2 in Fission Yeast.* For expression of the human cytochrome P450 in fission yeast, we used the strong *nmt1* promotor (*32*) in the pINT5 vector[2] or the weaker *nmt41* promotor in derivatives of the pREP41 vector (*33*). Strong expression of CYP11B2 using the *nmt1* promotor caused significantly slower growth of fission yeast (data not shown). Since an antibody against human CYP11B proteins was not available, we cloned the CYP11B2 cDNA into pREP41−Pk vectors, which allow immunological detection of the tagged proteins using a commercially available antibody. We transformed the P450 expression plasmids into fission yeast strains NCYC2036 and 1445 (all strains are listed in Table 1), induced P450 expression by depleting the media of thiamine, and analyzed cell lysates by SDS−PAGE and Western blot analysis. When the Pk tag was fused to the amino terminus of CYP11B2, we observed a single band which we assume to be the preprotein; in contrast, a carboxy terminally attached Pk tag led to the identification of two separate bands, which we assume to be the precursor protein and the mature protein, respectively (Figure 1a) and indicated that the presequence is only partially cleaved off after import. The analysis of mitochondrial lysates prepared from the same strains showed a strong enhancement of the immunological signal, indicating an exclusive targeting of the proteins with

[2] P. Wagner et al., unpublished results.

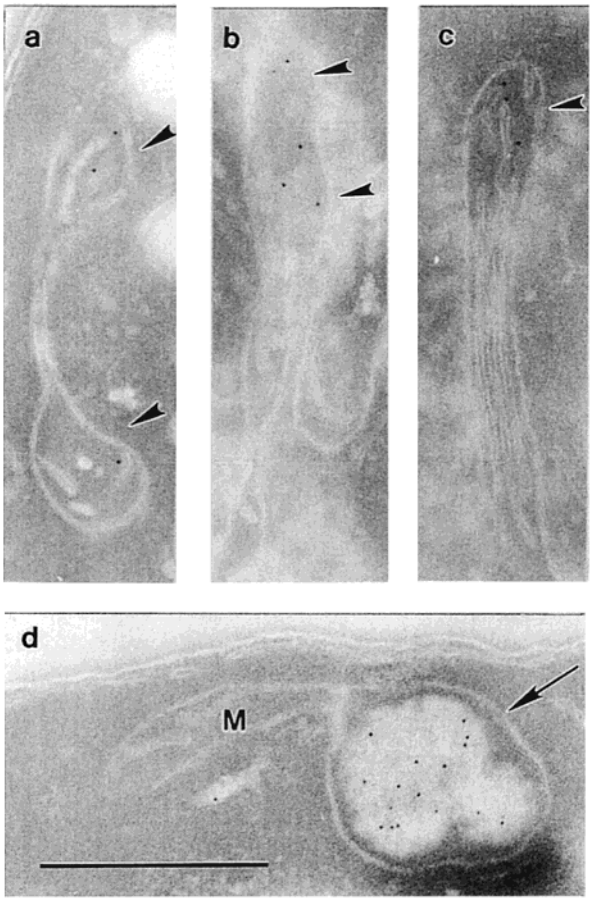


FIGURE 2: Detection of CYP11B2 in fission yeast mitochondria by immunoelectron microscopy. Strain MB193 was grown in the absence of thiamine, fixed, and processed for cryosectioning. Immunogoldstaining with α-PK Ab shows the localization of CYP11B2 inside the fission yeast mitochondria labeling the inner mitochondrial membranes (a−c; arrowheads) or in inclusion bodies enclosed by the outer mitochondrial membrane (d; arrow). M, mitochondrion. The bar represents 0.5 $\mu$m.

the CYP11B2 localization sequence into fission yeast mitochondria (not shown). Also, in these preparations, we found a third band of an apparently smaller size; the identity of this band is not clear yet. By comparison of total lysates of strain MB193 with a reference protein of know concentration in Western Blot analysis, we estimate an expression level of 2 nmol of CYP11B2 per liter of yeast culture (not shown). To further confirm the mitochondrial localization of the P450 enzyme, we tested for the immunocytochemical localization of human CYP11B2 by immunoelectron and fluorescence microscopy. It was found that immunogold staining viewed by electron microscopy localized CYP11B2 almost solely to the mitochondria (Figure 2, a−c). Interestingly, in some cells the expression of the P450 enzyme caused the formation of structures similar to inclusion bodies between the inner and the outer membrane of the mitochondria (Figure 2d). While we do not know the mechanism underlying this effect, we suppose that the change caused in the mitochondria could be accounting for the slow growth that we observed after strong induction of P450 expression. For fluorescence analysis, the CYP11B2 cDNA was cloned into plasmid pREP41-EGFP C (*20*), and both plasmids were transformed into fission yeast strain 1445 to yield strains MB241 (1445/pREP41-EGFP C) and MB249 (1445/pREP41−CYP11B2-EGFP C). Both strains were grown in the absence of thiamine, and living cells were examined by fluorescence microscopy. While strain MB241 did not show any significant fluorescence (Figure 3a), cells of strain MB249 (Figure 3b1−5) showed a typical mitochondrial fluorescence pattern (*34*). Together, these results clearly demonstrate that the human mitochondrial localization signal of CYP11B2 is functional in fission yeast: it targets the enzyme correctly to the organelle and is, at least partially, cleaved off after import into mitochondria, as is the case in mammalian cells.

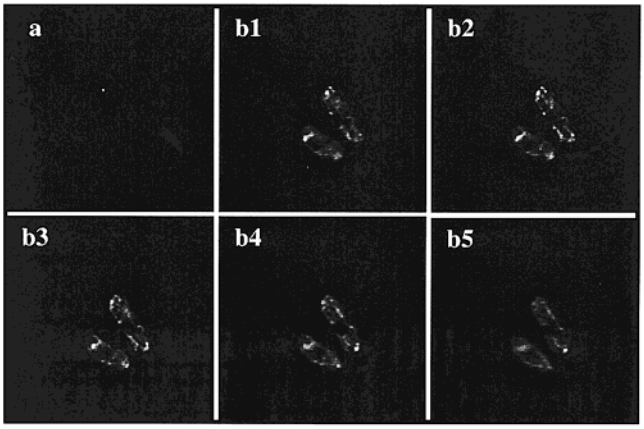


FIGURE 3: Detection of a CYP11B2-EGFP fusion protein in fission yeast mitochondria by fluorescence microscopy. Strains MB241 (expressing the EGFP fragment) and MB249 (expressing the CYP11B2-EGFP fusion protein) were grown in the absence of thiamine, and living cells were viewed by fluorescence microscopy. Shown are fluorescence images of MB241 cells (a) and a series of optical cuts of two representative MB249 cells (b1−b5).

*Fission Yeast Cells Expressing CYP11B2 Specifically Hydroxylate 11-Deoxycorticosterone and 11-Deoxycortisol.* Our initial objective was to obtain a suitable microorganism for the large scale expression of human CYP11B2, and, indeed, the P450 expression level achieved seems to be high enough to allow the isolation and in vitro investigation of the enzyme.[3] But having constructed fission yeast strains that express the steroid hydroxylase, it was tested whether the living yeast cells might show any activity toward the natural substrates of the P450 enzyme. For this purpose, the strain was cultivated in the absence of thiamine, and the steroid substrates were supplied to the indicated final concentrations with radioactively labeled substrates added to monitor the reactions. After steroid extraction and HPTLC analysis, we found an inducible production of cortisol, corticosterone, 18-OH-corticosterone, and aldosterone, respectively (Tables 2 and 3). While small amounts of steroid conversion were observed in transformed cells cultured with thiamine (probably due to the somewhat leaky promotor) wild-type fission yeast did not show any detectable steroid hydroxylase activity. Bioconversion of RSS to cortisol was accompanied by formation of a compound that in HPTLC comigrates with cortisone, which points to the presence of an intrinsic 11$\beta$-hydroxysteroid dehydrogenase activity in fission yeast (data not shown). Bioconversion of DOC under the conditions described led mainly to the production of corticosterone (Table 2) with much smaller amounts of 18-hydroxycorticosterone and aldosterone (Table 3). Only when most of the

[3] M. Bureik, unpublished results.

Table 2: Steroid Conversion by Fission Yeast Strains Expressing Human Mitochondrial Cytochromes P450 with or without Over-Expression of etp1 (etp1-OP)

| strain | expressed protein(s) | conversion of DOC to corticosterone [nmol/L * day][a] | conversion of RSS to cortisol [nmol/L * day][b] |
|---|---|---|---|
| 1445 | none | 0 | 0 |
| MB193 | CYP11B2 | 5.7 ± 1.5 | 1.5 ± 0.3 |
| MB224 | CYP11B2 and etp1-OP | 9.3 ± 1.3 | 2.8 ± 0.3 |

[a] Assayed by cultivating cells for 24 h in EMMG containing 100 nM DOC. [b] Assayed by cultivating cells for 24 h in EMMG containing 1 $\mu$M RSS. Data shown were calculated from three independent measurements.

Table 3: Comparison of Steroid Conversion by Fission Yeast Strain MB164 Expressing Human CYP11B2 during Fermentative and Respirative Growth

| growth conditions | corticosterone [nmol/L* day] | conversion of DOC to 18-hydroxy-corticosterone [nmol/L* day] | aldosterone [nmol/L* day] |
|---|---|---|---|
| fermentative[a] | 39 ± 3 | 4.4 ± 0.1 | 5.7 ± 0.1 |
| respirative[b] | 712 ± 123 | 20 ± 4 | 25 ± 5 |

[a] Assayed by cultivating cells for 24 h in EMM with 100 mg/L leucine and 0.2 $\mu$M DOC. [b] Assayed by cultivating cells for 24 h in EMMG with 100 mg/L leucine and 1 $\mu$M DOC. Data shown were calculated from three independent measurements.

DOC had vanished (after long term incubation) the formation of the latter two products increased (data not shown). This finding suggests that (i) CYP11B2 expressed in fission yeast preferably hydroxylates DOC and (ii) corticosterone produced by CYP11B2 dissociates from the enzyme too fast to be further hydroxylated. In a comparison of fission yeast strains grown under respirative or fermentative conditions, we observed that the rate of steroid hydroxylation was significantly higher when cells were grown under aerobic conditions using glycerol as a carbon source (Table 3). In a further experiment, we found that steroid hydroxylation activity of strain MB193 was not improved when cells were grown in media containing 1 mM 5-aminolevulinic acid (data not shown). This result indicates that endogenous heme biosynthesis is sufficient for formation of the CYP11B2 holoenzyme.

*Cloning, Sequencing, and Bioinformatic Analysis of etp1.* Since no coexpression of an electron transport protein was necessary to obtain strong P450 activity in CYP11B2 expressing fission yeast cells, an endogenous electron transport system must be present in *S. pombe*. In search of components of this system, we scanned the SWISS−PROT database (http://expasy.hcuge.ch/sprot/) and found a putative fission yeast protein that shows high homology toward Adx in its carboxy terminal part. We named this protein etp1 for *e*lectron *t*ransport *p*rotein 1. Its open reading frame (SPAC22E12.10c) is located on fission yeast chromosome I and had been sequenced by the *S. pombe* sequencing project at the Sanger Centre (ftp://ftp.sanger.ac.uk/pub/yeast/ sequences/pombe). We amplified this sequence by PCR from genomic DNA and cloned it into pMOSBlue (Amersham). Sequencing of several independent clones revealed several differences in comparison to the Sanger sequence: The nucleotide exchanges C280T, T335C, and C1328T lead to the following changes in the deduced amino acid sequence: Pro94Ser, Ile112Thr, and Ala443Val. Additionally, we found two silent mutations (T318C and T840C). Homology analysis of the etp1 protein showed that it is comprised of domains that have been separately found on other proteins of known function: it contains a large domain in the amino terminus and the middle part of the protein (amino acids 85 to 473) that has significant homology to the COX15 proteins of humans (*35*) and bakers yeast (*36*), which function in the assembly of the COX complex (Figure 4). This domain contains eight potential transmembrane regions. By contrast, the carboxy terminal part of etp1 shows a high homology to the members of the ferredoxin family of electron transport proteins (Figure 5). It shares a homology of more than 40% in its last 124 amino acids with the human Adx. The etp1 protein contains 631 amino acids and is therefore much larger than all other members of the Adx family. Thus, the etp1 protein contains domains that have so far not been found together on any other polypeptide. We designated the two parts of the protein etp1$^{cd}$ and etp1$^{fd}$. A comparison between the predicted protein sequence for etp1$^{fd}$, mammalian adrenodoxins (mature forms), bacterial [2Fe-2S] ferredoxins, and the budding yeast Adx homologue YAH1 (*37*) demonstrated that both yeast proteins are significantly more closely related to adrenodoxins than are all bacterial ferredoxins (Figure 6). Etp1 contains four cysteine residues conserved among ferredoxins (Cys-556, Cys-562, Cys-565, and Cys-602) that participate in the formation of the iron−sulfur cluster (*38*); also, two aspartic acid residues (Asp-76 and Asp-79) that are necessary for interaction of Adx with AdR (*39*) are conserved in etp1 (Asp-586 and Asp-589), as well as the highly conserved and structurally important Pro-108 in mature bovine Adx (*40*), which probably corresponds to residue Pro-618 in etp1. Taken together, these data support the view that etp1 is more closely related to mammalian adrenodoxins than any other protein of an unicellular organism.

*Overexpression of etp1 Enhances P450 Activity in CYP11B2 Expressing Fission Yeast Cells.* To support the hypothesis that the CYP11B2-dependent actvity in recombinant fission yeast is in fact mediated by etp1, this protein was overexpressed, and steroid hydroxylation activity was measured in the resulting strain. For overexpression of etp1, we cloned the etp1 cDNA into vector pREP42 and transformed this construct into strains MB193 (CYP11B2) to yield strain MB224 (CYP11B2/etp1-OP). When testing total protein lysates of these strains by SDS−PAGE and Western blot analysis using a specific antibody generated against bacterially expressed etp1$^{fd}$, the etp1 protein was hardly detected at its predicted mass of 70 kDa (not shown), while the level of CYP11B2 expression in these strains was not affected by overexpression of etp1 (Figure 1b). However, after preparation of mitochondria the etp1 overexpressing strain showed a strong increase in the etp1 protein level, demonstrating that etp1 is indeed imported in this organelle (Figure 7). The predominant signal obtained from mitochondrial fission yeast lysates migrates at about 30 kDa, indicating a truncated form of etp1 and thus the cleavage of the etp1 protein probably by mitochondrial peptidases. The activity of a MPP and a MIP has been demonstrated in *S. pombe* mitochondria (*41*), and several putative recognition motives typical of processing

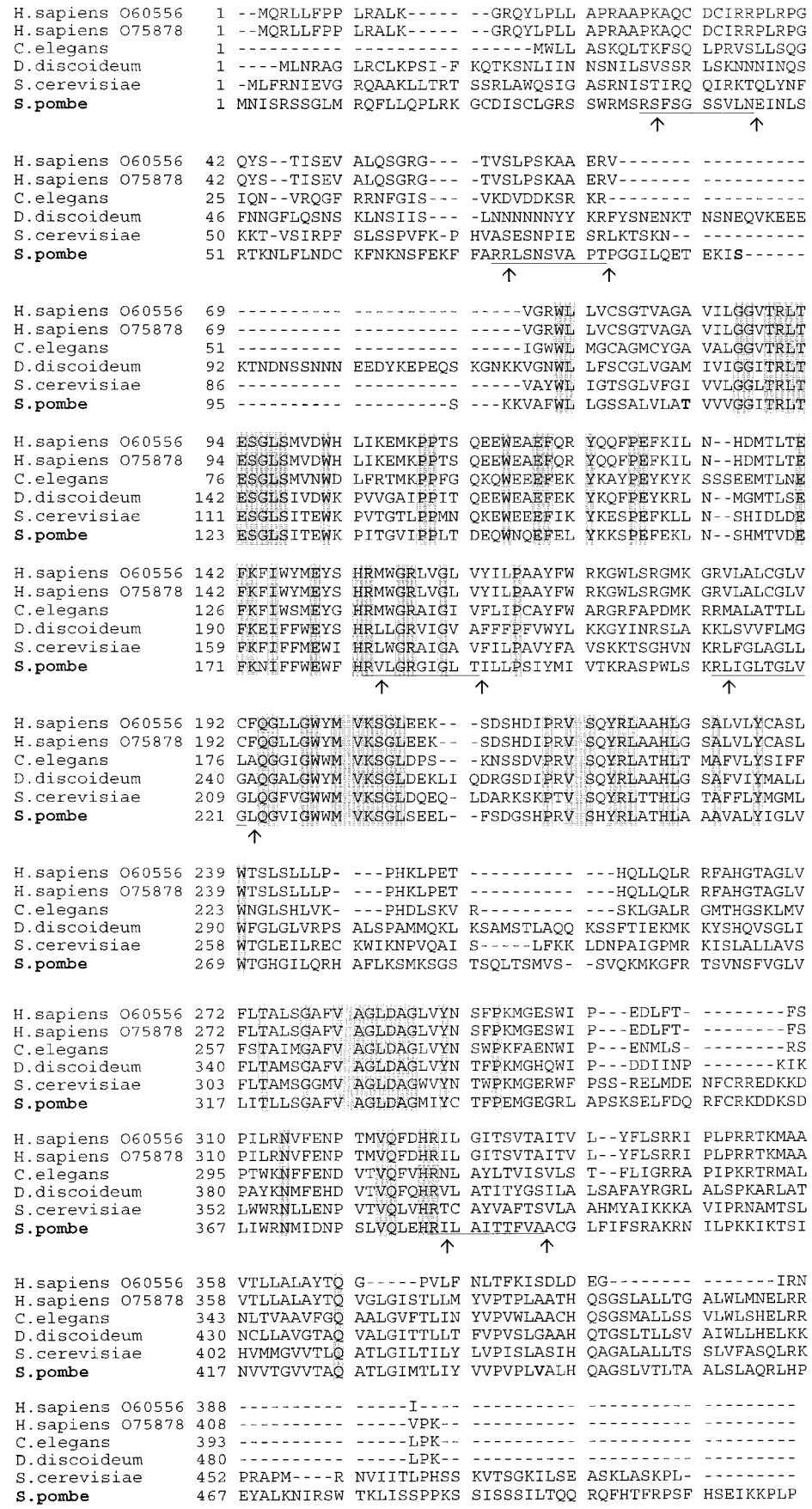


FIGURE 4: Comparison of the deduced amino acid sequence for budding yeast COX15, etp1, and putative COX15 homologues from other species. For this alignment, amino acids 1 to 515 of etp1 were used; the two amino acids found in this study to differ from the Sanger sequence are shown in bold. Shared sequence identities between all proteins are *shaded*. Deduced recognition sequences − RX(↑)(F/L/I)-XX(T/S/G)XXXX(↑) − for mitochondrial endopeptidases MPP and MIP are underlined and potential cleavage sites are indicated by arrows.

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H.sapiens      61 SSS------- ------EDKI TVHFI-NRDG ETL--TTKGK VGDSLLDVVV
S.scrofa       59 SSS------- ------EDKI TVHFI-NRDG KTL--TTQGK VGDSLLDVVI
M.musculus     65 SSS------- ------EDKI TVHFK-NRDG ETL--TTKGK IGDSLLDVVI
O.aries         1 SSS------- ------EDKV TVNFI-NRDG ETL--TTKGK VGDSLLDVVV
R.norvegicus   65 SSS------- ------EDKV TVHFK-NRDG ETL--TTKGK VGDSLLDVVI
B.taurus       59 SSS------- ------EDKI TVHFI-NRDG ETL--TTKGK IGDSLLDVVV
G.gallus       20 CSS------- ------EDKI TVHFI-NRDG DKL--TAKGK PGDSLLDVVV
S.pombe       516 ---------- ------GTGI KVFFV-TPEG REI--MIEGN EGDSILDLAH
S.cerevisiae   42 STSSFLNHGH LKKPKPGEEL KITFI-LKDG SQK--TYEVC EGETILDIAQ
H.influenzae    1 ---------- ---------P KVIFLPNEDF CPEGMVVDAA TGDNLLEVAH
Pseudomo.sp.    1 ---------- ---------P RVVFIDEQSG EYA---VDAQ DGQSLMEVAT
C.crescentus    1 ---------- --------MA KITYI-QHDG AEQ--VIDVK PGLTVMEGAV
E.coli          1 ---------- ---------P KIVILPHQDL CPDGAVLEAN SGETILDAAL
Rhodococcus     1 ---------- ---------P TVTYV-HPDG TKH--EVEVP TGKRVMQAAI
R.capsulatus    1 ---------- ---------A KIIFI-EHNG TRH--EVEAK PGLTVMEAAR
P.putida        1 ---------- ---------S KVVYV-SHDG TRR--ELDVA DGVSLMQAAV

H.sapiens      95 ENNLDIDGFG ACEGTLACST CHLIFEDHIY EKLDAITDEE NDMLDLAYGL
S.scrofa       93 ENNLDIDGFG ACEGTLACST CHLIFEDHIF EKLEAITDEE NDMLDLAYGL
M.musculus     99 ENNLDIDGFG ACEGTLACST CHLIFEDHIY EKLDAITDEE NDMLDLAFGL
O.aries        35 ENNLDIDGFG ACEGTLACST CHLIFEQHIY EKLEAITDEE NDMLDLAYGL
R.norvegicus   99 ENNLDIDGFG ACEGTLACST CHLIFEDHIY EKLDAITDEE NDMLDLAFGL
B.taurus       93 QNNLDIDGFG ACEGTLACST CHLIFEQHIF EKLEAITDEE NDMLDLAYGL
G.gallus       54 ENNLDIDGFG ACEGTLACST CHLIFEDHIF EKLDAITDEE MDMLDLAYGL
S.pombe       547 ANNIDLEG-- ACEGSVACST CHVIVDPEHY ELLDPPEEDE EDMLDLAFGL
S.cerevisiae   89 GHNLDMEG-- ACGGSCACST CHVIVDPDYY DALPEPEDDE NDMLDLAYGL
H.influenzae   32 NAGVEIHH-- ACDGSCACTT CHVIVREG-F DSLNETSDQE EDMLDKAWGL
Pseudomo.sp.   29 QNGVPGIV-A ECGGSCVCAT CRIEIEDAWV EIVGEANPDE NDLLQSTGEP
C.crescentus   30 KNNVPGID-A DCGGACACAT CHVYVDEAWL DKTGDKSAME ESMLDFAENV
E.coli         32 RNGIEIEH-- ACEKSCACTT CHCIVREG-F DSLPESSEQE DDMLDKAWGL
Rhodococcus    29 GAGIDGIV-A ECGGQAMCAT CHVYVESPWA DKFPSISEEE DEMLDDTVSP
R.capsulatus   29 DNGVPGID-A DCGGACACST CHAYVDPAWV DKLPKALPTE TDMIDFAYEP
P.putida       29 SNGIYDIV-G DCGGSASCAT CHVYVNEAFT DKVPAANERE IGMLECVTAE

H.sapiens     145 TDR-SRLGCQ ICLTKSMDNM TVRVPETVAD ARQSIDVGKT S----
S.scrofa      143 TDR-SRLGCQ ICLTKAMDNM TVRVPEAVAD ARESIDLGKN SSKLE
M.musculus    149 TDR-SRLGCQ VCLTKAMDNM TVRVPEAVAD VRQSVDMSKN S----
O.aries        85 TDR-SRLGCQ ICLTKAMDNM TVRVPDAVSD ARESIDMGMN SSKIE
R.norvegicus  149 TNR-SRLGCQ VCLTKAMDNM TVRVPEAVAD VRQSVDMSKN S----
B.taurus      143 TDR-SRLGCQ ICLTKAMDNM TVRVPDAVSD ARESIDMGMN SSKIE
G.gallus      104 TET-SRLGCQ ICLKKSMDNM TVRVPEAVAD ARQSVDLSKN S----
S.pombe       595 EET-SRLGCQ VLLRKDLDGI RVRIPAQTRN IRLERPKA-- -----
S.cerevisiae  137 TET-SRLGCQ IKMSKDIDGI RVALPQMTRN VNNNDFS--- -----
H.influenzae   79 EMD-SRLSCQ CVVGNE-D-L VVEIPKYNLN HANEAAH--- -----
Pseudomo.sp.   78 MTAGTRLSCQ VFIDPSMDGL IVRVPLPA-- ---------- -----
C.crescentus   79 EPN-SRLSCQ IKVSDALDGL VVRLPESQH- ---------- -----
E.coli         79 EPE-SRLSCQ ARVTDE-D-L VVEIPRYTIN HAREH----- -----
Rhodococcus    78 RTEASRLSCQ LVVSDDVDGL IVRLPEEQV- ---------- -----
R.capsulatus   78 NPATSRLTCQ IKVTSLLDGL VVHLPEKQI- ---------- -----
P.putida       78 LKPNSRLCCQ IIMTPELDGI VVDVPDRQW- ---------- -----
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FIGURE 5: Comparison of mammalian, yeast, and bacterial [2Fe-2S] ferredoxins. Mitochondrial location sequences of adrenodoxins were omitted. For this alignment, amino acids 516 to 631 of etp1 were used. Shared sequence identities between the studied proteins are *shaded*.

by MPP and MIP are present in the etp1 sequence (Figure 4). For comparison, the apparent molecular weight of in vitro translated etp1 and etp1$^{cd}$ was determined by SDS−PAGE and autoradiography (Figure 8): full-length etp1 displayed a molecular mass of about 80 kDa, while the mutant containing only the COX15 domain had an apparent size of 55 kDa. This difference in apparent molecular weight between full-length etp1 and etp1$^{cd}$ suggests an apparent molecular weight of approximately 25 kDa for etp1$^{fd}$. We conclude from these data that the carboxy terminal part of etp1 including the complete ferredoxin domain and a number of amino acids between this domain and the protease site is cleaved off after import into mitochondria. This process is observed both with and without overexpression of etp1 (Figure 7; compare lanes 1 and 3 to lanes 2 and 4). To elucidate the influence of etp1 overexpression on the activity of CYP11B2, we measured steroid conversion by this fission yeast strain (MB224) and found an increase in steroid hydroxylase activity of 63 and 87% as compared to strain MB193 using DOC and RSS as substrates, respectively (Table 2). This finding strongly suggests that the etp1 protein (or more precisely: its ferredoxin domain) is indeed conferring electrons on this human mitochondrial P450 enzyme in living fission yeast cells.

*Recombinant etp1 Can Transfer Electrons from AdR to CYP11B1 and CYP106A2 in Vitro.* To test whether recombinant etp1$^{fd}$ can transfer electrons from AdR to a mitochondrial P450 in vitro, it was expressed in *E. coli* and purified (as described in experimental procedures). In a reconstituted assay consisting of bovine AdR and bovine CYP11B1, the conversion of DOC to corticosterone was monitored at different concentrations of either bovine mature Adx or etp1$^{fd}$. From the results of these measurements, we determined the $K_M$-values of Adx and etp1$^{fd}$ to be 2.39 ± 0.12 $\mu$M and 2.32 ± 0.30 $\mu$M, respectively, while the turnover numbers were found to be 17.1 ± 2.5 $s^{-1} \times 10^{-3}$ in case of etp1$^{fd}$ and 70.0 ± 2.3 $s^{-1} \times 10^{-3}$ in case of Adx. These results demonstrate that purified recombinant etp1$^{fd}$ can transfer electrons from AdR to CYP11B1 in vitro, although with lower efficiency than bovine Adx. A similar experiment was done to determine whether etp1$^{fd}$ can transfer electrons on a bacterial cytochrome P450 as well. For this purpose, we tested conversion of DOC to 15-hydroxy-11-deoxycorticosterone catalyzed by CYP106A2 from *Bacillus megaterium* strain ATCC 13368 in the presence of AdR and Adx or etp1$^{fd}$. From these experiments, we calculated a $K_M$

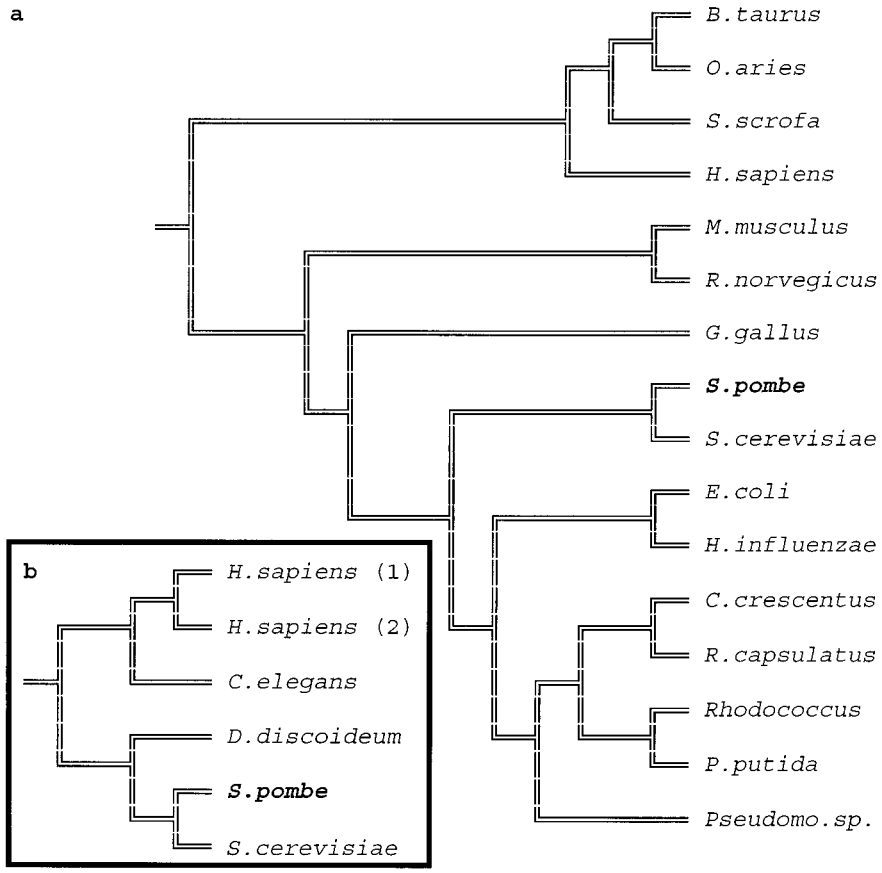


FIGURE 6: Phylogenetic trees of mammalian, yeast, and bacterial [2Fe-2S] ferredoxins (a) and of etp1$^{cd}$, budding yeast COX15, and putative COX15 homologues from other species (b). Mitochondrial location sequences of adrenodoxins were omitted. For the alignment with ferredoxins, amino acids 516 to 631 of etp1 were used, while the first 515 amino acids of etp1 were used in the alignment with COX15 proteins.

value of $4.79 \pm 0.25\ \mu$M for the reaction with Adx and $1.13 \pm 0.46\ \mu$M for etp1$^{fd}$; the turnover numbers were calculated to be $9.9 \pm 0.9\ s^{-1} \times 10^{-3}$ using Adx and $2.9 \pm 0.4\ s^{-1} \times 10^{-3}$ using etp1$^{fd}$. Therefore, etp1$^{fd}$ can also transfer electrons to CYP106A2 in vitro. These experiments show for the first time a yeast ferredoxin to productively interact with mammalian and bacterial cytochromes P450.

## DISCUSSION

The use of unicellular organisms for the investigation and purification of the mitochondrial P450s CYP11B1 and CYP11B2 has proven to be more difficult than in the case of microsomal P450s. Therefore, most investigations on these two enzymes were done using adrenal tissues or mammalian expression systems. The expression of bovine CYP11B1 in *S. cerevisiae* has been reported but did not yield enzyme quantities sufficiently high for purification (*16*). In search of an alternative model organism, we choose to test the fission yeast *S. pombe* for mainly two reasons: (i) the strong and functional expression of the microsomal P450s CYP2C11 (*14*) and CYP2C9 (*15*) in fission yeast had been demonstrated, and in a direct comparison study the results for fission yeast expression were superior to expression in bakers yeast (*15*). (ii) the intracellular membrane systems of *S. pombe* are more highly developed as compared to those of *S. cerevisiae* (*42*), and although it belongs to fungi, fission yeast has many distinct features in its mitochondria far removed from other fungi (*43*). In contrast to filamentous fungi, no endogenous steroid hydroxylation activity has been observed in either *S. cerevisiae* or *S. pombe*; however, several hydroxysteroid dehydrogenase activities have been detected in both yeasts (*44*, and references therein). This lack of endogenous steroid hydroxylase activity renders both yeasts attractive hosts for artificial steroid metabolism driven by mammalian cytochromes P450. To test *S. pombe* as a model system for the study of mitochondrial cytochrome P450-enzymes, we expressed human CYP11B2 in this yeast. In general, a protein may be imported into mitochondria provided it has an amino terminal targeting sequence rich in positively charged amino acid residues (*45*, *46*), although internal targeting signals have been reported, too (*47*, *48*). Other prerequisites of the targeting process are chaperoning factors that ensure a loosely folded, transport-competent conformation and the accessibility of the signals, and membrane localized translocation machineries (*49*). Finally, the imported preprotein undergoes proteolytic processing by specific maturases that reside in the mitochondrial matrix. In this work, we demonstrate that human CYP11B2 expressed in fission yeast almost exclusively localizes to mitochondria, as was shown by electron microscopy (Figure 2) and by fluorescence of CYP11B2-GFP fusion proteins (Figure 3). This is to our knowledge the first example of a human mitochondrial targeting sequence being functional in fission yeast. Since mitochondrial targeting of bovine CYP11B1 (which has a very similar presequence) expressed in bakers yeast is only efficient if the original presequence is replaced by an amino terminal COX6 prepeptide (*16*), the functionality of this targeting process seems to distinguish

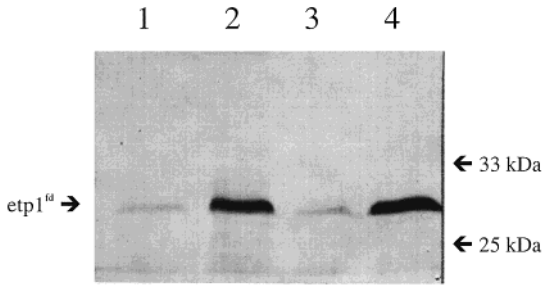


FIGURE 7: Immunologic detection of etp1 in *S. pombe*. Fission yeast strains were grown in the absence of thiamine, and mitochondrial protein lysates were prepared and analyzed by SDS−PAGE and Western blotting using an etp1-specific antibody. Lane 1, MB223 (wt); lane 2, MB226 (overexpressing etp1); lane 3, MB193 (expressing CYP11B2); lane 4, MB224 (expressing CYP11B2 and overexpressing etp1). Overexpression of full-length etp1 cDNA leads to a strong enhancement in the amount of the detected etp1 fragment. The apparent size of the detected protein indicates that the etp1 carboxy terminal region is cleaved off after import into mitochondria.

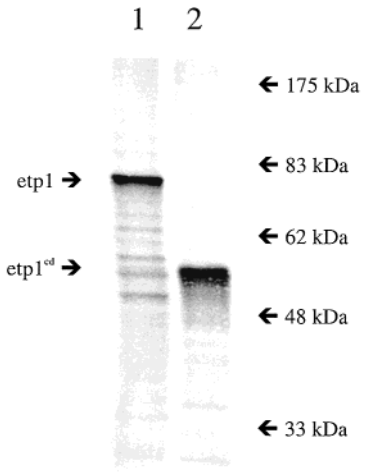


FIGURE 8: In vitro translation of etp1. In vitro translation reactions with [$^{35}$S]methionine were performed using either plasmid pMB131 encoding full-length etp1 (lane 1) or plasmid pMB132 encoding the carboxy terminal deletion mutant etp1$^{1-515}$ (lane 2). Reaction products were separated by 10% SDS−PAGE, blotted onto nitrocellulose, and analyzed by autoradiography.

*S. pombe* from *S. cerevisiae*. In opposition to the efficient targeting, cleavage of the CYP11B2 presequence after mitochondrial import is much less effective, which is indicated by the strong preprotein signal found by Western blot analysis (Figure 1).

Fission yeasts expressing CYP11B2 display the ability to convert in vivo DOC to corticosterone, 18-hydroxycorticosterone, and aldosterone as well as RSS to cortisol, respectively. Although mitochondrial P450 steroid hydroxylases depend for their activity on an electron transport chain that consists of the two proteins Adx and AdR, no coexpression of these proteins is needed for efficient substrate conversion by intact fission yeast cells. This result was unexpected, since fission yeast contains no mitochondrial P450 enzyme. Moreover, after expression of mammalian mitochondrial P450 enzymes in *S. cerevisiae*, steroid hydroxylase activity is almost undetectable in vivo without coexpression of Adx (*16*). A database search revealed the presence of an ORF on chromosome I of fission yeast (which we have named etp1) that shows in its carboxy terminal domain high homology toward ferredoxins, while the rest of the protein is highly homologous to a cytochrome *c* oxidase assembly factor, COX15. Thus, this protein combines domains that have so far not been found together on any other polypeptide, and containing 631 residues it is much larger in size than all other known members of the Adx family. Using an antibody raised against etp1$^{fd}$, we investigated etp1 expression both in wild-type fission yeast and in etp1 overexpressing strains (Figure 7). We were unable to detect full-length etp1 in total protein lysates or mitochondrial lysates of these strains. However, in mitochondrial lysates, we found a protein band that had an apparent size of 30 kDa in SDS−PAGE. Since this band specifically reacted with our anti-etp1$^{fd}$ antibody and its intensity was significantly increased after overexpression of full-length etp1, we conclude that a carboxy terminal part of etp1 including the ferredoxin domain must be very efficiently cleaved from the COX15 domain after mitochondrial import. The identification of potential protease cleavage sites in the etp1 primary sequence is in agreement with this hypothesis. Although the amount of full-length etp1 was too small to be identified, we assume that full-length etp1 is synthesized and imported into mitochondria as a precursor protein for two reasons: (i) The etp1$^{cd}$ polypeptide is a COX15 homologue that can be expected to fulfill a function inside the mitochondria and contains a presequence that according to network-based targeting prediction tools is very probably a mitochondrial localization sequence: The program Mitoprot (http://mips.gsf.de/cgi-bin/proj/medgen/mitofilter) predicted a probability of 0.9735 for export into mitochondria while the program TargetP (http://www.cbs.dtu.dk/services/TargetP/) calculated a score of 0.911 for targeting to the same organelle; therefore, there is no reason to assume a nonmitochondrial localization of etp1$^{cd}$. On the other hand, the mitochondrial localization of etp1$^{fd}$ was demonstrated by us as described above. So, presuming both domains of etp1 are located inside the mitochondria, it would be rather expected that processing of the etp1 precursor does not take place ahead of mitochondrial import. (ii) In addition, full-length etp1 could be amplified by PCR from a fission yeast cDNA library (data not shown) and in vitro translation demonstrated the synthesis of full-length etp1 (Figure 8). Hence, more work will be necessary to clarify the details of etp1 synthesis and processing, but this is beyond the scope of this current study.

As expected, overexpression of etp1 lead to a significant increase of in vivo steroid hydroxylase activity. Moreover, steroid hydroxylase activity was higher under respirative as compared to fermentative conditions. It can be assumed that etp1, which probably has a function in the respiratory chain, either displays higher activity under these conditions and/or receives more electrons from this cascade, which in turn can then be transferred onto CYP11B2. In addition, steroid hydroxylation requires oxygen as a cosubstrate, which could also explain this effect. It would be expected that after gene disruption of etp1 steroid hydroxylase activity of CYP11B2 in fission yeast is lost. However, the *S. cerevisiae* ferredoxin Yah1, which recently has been shown to function in the biogenesis of iron−sulfur proteins (*50*), is essential for bakers yeast viability (*37*). Therefore, we did not expect an etp1 deletion mutant to be viable and did not try to create such a mutant. Instead, we demonstrated that the bacterially expressed and purified ferredoxin domain of etp1 could replace

bovine Adx in a reconstituted steroid hydroxylase assay, thereby interacting productively with its bovine redox partners AdR and CYP11B1. This experiment proves that the etp1 ferredoxin domain is capable of transferring electrons on mammalian cytochromes P450 of the CYP11B family, although not as efficient as bovine Adx. Moreover, we showed that etp1$^{fd}$ can also reduce the bacterial cytochrome P450 CYP106A2 in vitro. Until now, it had been shown that mammalian adrenodoxin can transfer electrons on mammalian mitochondrial and some bacterial cytochromes P450, while ferredoxins of unicellular species were reducing only bacterial, but not mitochondrial P450s. Here we show that the fission yeast etp1 protein is the first ferredoxin of an unicellular organism that is capable of productively interacting with P450s of both bacterial and mammalian mitochondrial origin.

COX, the terminal component of the mitochondrial respiratory chain, is a multiheteromeric enzyme embedded in the mitochondrial inner membrane. From a genetic point of view, COX results from the complementation of two separate genetic systems, the nuclear genome and the mitochondrial genome (*51*). In humans, COX is composed of 13 subunits: the three largest are encoded by mtDNA genes, while the remaining subunits are encoded by nuclear genes (*52*). In addition to encoding most of the protein subunits of the respiratory complexes, the nuclear genome codes for many proteins that function either in the expression of mitochondrial genes or in the assembly and function of respiratory proteins (*53*, *54*). Budding yeast COX15 seems to belong to this group, since studies on COX15 mutants did not reveal any effects on the expression of the mitochondrial or nuclear COX genes, or on mitochondrial copper metabolism or heme *a* biosynthesis. COX15 may have a role in COX assembly, since all subunits are synthesized normally but are unstable in a COX15 null mutant (*36*). The gene coding for the human homologue of the budding yeast COX15 protein, h-COX15, has been identified and the presence of several transcripts from this gene were demonstrated, with the deduced protein sequences diverging in a short region in the carboxy terminus (*33*). However, the functional role of the two h-COX15 isoforms remains to be elucidated. Our results suggest that the carboxy terminal part of etp1 is cleaved off after import into mitochondria; this part of the protein is probably released into the matrix, while the amino terminal COX15 domain, containing a number of potential transmembrane regions, possibly remains in the inner mitochondrial membrane and fulfills function similar to that of bakers yeast COX15. This, however, remains to be shown. Still, to our knowledge this is the first example of a single fission yeast precursor protein to be cleaved into two distinct functional proteins. It is worth emphasizing that among all species with complete genome sequences available so far there is no complete etp1 homologue containing both the COX15 and the ferredoxin domain. This could indicate that etp1 is either an evolutionary very old protein or, quite opposite, that it is the product of a special development that for some reason took place only in fission yeast. Although the genome of *S. pombe* is almost completely sequenced by now, it is not yet clear whether it is a fast-evolving organism or not (as compared for example to *S. cerevisiae*) or even if such a general statement for the whole genome can be made.[4] However, there are reasons to assume a slower evolutionary rate for the *Schizosaccharomyces* lineage than for the *Saccharomyces* branch (*55*).

It has been suggested that all eukaryotic cytochrome P450 enzymes might stem from a common CYP51 ancestor and that the mitochondrial P450s could have evolved from microsomal P450s that were accidently targeted to mitochondria and found a suited electron transport system present in this organelle (*56*). While there is no eukaryote known to contain a single P450 species, *S. pombe* contains only two (CYP51 and CYP61). Therefore, this yeast might reflect an evolutionary very old situation: an unicellular eukaryote with no mitochondrial P450s present, but already containing a mitochondrial electron transport system capable of supporting these enzymes. In this context, it is interesting to mention that bakers yeast does not contain a similar electron transport system, which is supported by the observation that *S. cerevisiae* cells expressing bovine CYP11B1 but not Adx show very low steroid hydroxylase activity in vivo (*16*). It is intriguing to speculate that the origin of the mitochondrial P450 electron transport chain might be tracked back to components of the respiratory chain, highlighted by etp1 which contains a COX15 domain and a ferredoxin domain on the same polypeptide. The revelation of the functions of etp1 in fission yeast might add a new perspective to the functions of mammalian adrenodoxins. We have not yet investigated how the etp1 protein itself is reduced. There is an open reading frame in the fission yeast genome (SPBC3B8.01c) that codes for a putative protein with significant homology to mammalian adrenodoxin reductases. When testing this putative protein using above-mentioned targeting prediction tools, high scores for mitochondrial localization were obtained. Thus, it is very well possible that in fission yeast mitochondria electrons are transferred from NAD(P)H via the SPBC3B8.01c-encoded protein to etp1; however, so far no experiments were done in our lab to study these mechanisms. Also, the endogenous role of this electron transport chain remains obscure since, as mentioned above, the presence of mitochondrial cytochromes P450 can be ruled out. Therefore, it is not possible at the moment to describe the mechanism of electron flow from NAD(P)H to etp1 in *S. pombe* except for speculating that the putative NAD(P)H-etp1 reductase is reduced by NADH or NADPH and transfers electrons on etp1.

[4] M. Sipiczki, personal communication.

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