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Expression and purification of recombinant *Saccharomyces cerevisiae* mitochondrial carrier protein YGR257Cp (Mtm1p)

Mei M. Whittaker and James W. Whittaker*

Institute for Environmental Health, Division of Environmental and Biomolecular Systems, Oregon Health & Science University, 3181 SW Sam Jackson Park Road, Portland, OR 97239-3098, United States

Abstract

The *Saccharomyces cerevisiae* mitochondrial carrier YGR257Cp (Mtm1p) is an integral membrane protein that plays an essential role in mitochondrial iron homeostasis and respiratory functions, but its carrier substrate has not previously been identified. Large amounts of pure protein are required for biochemical characterization, including substrate screening. Functional complementation of a *Saccharomyces* knockout by expression of TwinStrep tagged YGR257Cp demonstrates that an affinity tag does not interfere with protein function, but the expression level is very low. Heterologous expression in *Pichia pastoris* improves the yield but the product is heterogeneous. Expression has been screened in several *Escherichia coli* hosts, optimizing yield by modifying induction conditions and supplementing with rare tRNAs to overcome codon bias in the eukaryotic gene. Detection of an additional N-terminal truncation product in *E. coli* reveals the presence of a secondary intracistronic translation initiation site, which can be eliminated by silent mutagenesis of an alternative (Leu) initiation codon, resulting in production of a single, full-length polypeptide (~30% of the total protein) as insoluble inclusion bodies. Purified inclusion bodies were successfully refolded and affinity purified, yielding approximately 40 mg of pure, soluble product per liter of culture. Refolded YGR257Cp binds pyridoxal 5'-phosphate tightly ($K_D < 1 \mu\text{M}$), supporting a new hypothesis that the mitochondrial carrier YGR237Cp and its homologs function as high affinity PLP transporters in mitochondria, providing the first evidence for this essential transport function in eukaryotes.

Keywords

Mitochondrial carrier protein; Integral membrane protein; Initiation codon; Codon bias; Rare tRNA; Pyridoxal 5'-phosphate

Introduction

Mitochondrial carrier proteins (MCPs)¹ [1,2] are integral membrane proteins that serve as essential pathways for the exchange of metabolites across the otherwise impermeable inner mitochondrial membrane in eukaryotic cells, connecting metabolic processes in two subcellular compartments, the cytosol and the mitochondrial matrix. The number of MCPs is small compared to the total number of metabolites in a living cell: 34 MCPs have been identified in the *Saccharomyces cerevisiae* genome based on the characteristic tripartite

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*Corresponding author: Fax: +1 503 346 3427. jim@ebs.ogi.edu (J.W. Whittaker).

¹Abbreviations used: MCP, mitochondrial carrier protein; BCA, bicinchoninic acid; DTNB, 5,5'-dithiobis 2-nitrobenzoic acid; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; TCEP, tris(2-carboxyethyl)phosphine; DOC, deoxycholate; CMC, critical micelle concentration; MAB, monoclonal antibody; PLP, pyridoxal 5'-phosphate.

sequence motif. MCPs undergo an eversion cycle, binding substrates and facilitating transport down a chemical gradient (in uniport mode operation) or coupling the chemical gradients of two substrates (in symport or antiport mode). Tripartite MCPs play important roles in metabolism, including exchange of ATP, ADP and phosphate [3], transfer of biosynthetic precursors (e.g., dicarboxylic acids, citrate) [4]; and high affinity uptake of metal ions (e.g., Fe^{2+}) [5] and other essential nutrients. Many of the yeast MCPs have been assigned functions based on deletion phenotype or direct functional assay of the purified protein [6–8]. Recombinant expression is generally required for molecular characterization, since the native expression level in mitochondria tends to be very low. However, recombinant expression levels are quite variable [8], and expression of some of the yeast MCPs has never been reported.

The *S. cerevisiae* genomic locus YGR257C encodes an MCP that has been the subject of genetic and metabolic studies for more than a decade [9–13]. YGR257Cp was originally identified as a manganese ion transporter and chaperone [9] based on a deletion phenotype that included a defect in metal activation of mitochondrial manganese superoxide dismutase (Sod2p) which served as the basis for the gene name (mtm1, manganese trafficking factor for mitochondrial Sod2p). However, later studies have raised questions about that assignment [10], suggesting a more direct role in mitochondrial iron homeostasis involving iron–sulfur cluster assembly and heme biosynthesis. These earlier studies show that deletion of the gene results in irreversible loss of mitochondrial function and a rho-minus respiratory defect, but have been unable to define the true biological function of YGR257Cp. Homologs in higher organisms (*Arabidopsis*, AtMtm1p [11]; zebrafish, SLC25A39p [12]) have been reported to play important roles in antioxidant defense and regulation of heme biosynthesis, respectively. We have undertaken recombinant expression of YGR257Cp in *S. cerevisiae*, *Pichia pastoris*, and *Escherichia coli*, optimizing expression level and refolding in order to support direct biochemical characterization of the pure protein. The approaches used here may be generally useful in preparation of other recombinant MCPs.

Materials and methods

Biological materials

Designer deletion strains of *S. cerevisiae* [14] (*S. cerevisiae* BY4741 MATa his3 Δ 1 leu2 Δ 0 met15 Δ 0 ura3 Δ 0 ygr257c::KanMX4, and *S. cerevisiae* BY4739 MAT α leu2 Δ 0 lys2 Δ 0 ura3 Δ 0 and *S. cerevisiae* BY4700 MATa ura3 Δ 0) are from the American Type Culture Collection (Bethesda, MD). *S. cerevisiae* INVSc1 (MATa his3 Δ 1 leu2 trp1–289 ura3–52) and *P. pastoris* X33 are from Invitrogen (Grand Island, NY). *E. coli* BL21(DE3), *E. coli* BL21 Star (DE3), and *E. coli* BL21(DE3) CodonPlus RIL are from Stratagene (La Jolla, CA). The Walker strain C41(DE3) [15] is from Lumigen (Ann Arbor, MI).

Culture media

Bacterial cultures were routinely maintained on Luria–Bertani (LB) medium (0.5% yeast extract, 0.5% NaCl; 1% tryptone) with appropriate antibiotic selection (carbenicillin, 125 $\mu\text{g}/\text{mL}$; chloramphenicol, 35 $\mu\text{g}/\text{mL}$). Super Optimal Broth with Catabolite repression (SOC) medium (0.5% yeast extract, 0.5% NaCl, 2% tryptone, 1 mM MgSO_4 , 0.2% glucose) was used for recovery after bacterial transformation. Studier [16] catabolite repression medium (ZYPG) (0.5% yeast extract, 1% N–Z amine, 1 $\times$ NPS, 0.2% glucose) was used with appropriate antibiotics to select transformants. The Studier method for autoinduction [16] was used for protein expression in ZYP-5052 medium (ZYP with 0.5% glycerol, 0.05% glucose, 0.2% α -lactose) supplemented with 125 $\mu\text{g}/\text{mL}$ carbenicillin and 25 $\mu\text{g}/\text{mL}$ chloramphenicol.

Yeast cultures were routinely maintained on YPD medium (1% yeast extract, 2% peptone, 2% glucose) [17]. Presporulation (PSP2) medium [18] (0.1% yeast extract, 0.67% yeast nitrogen base without amino acids, 1% potassium acetate) and 1% KAc medium (1% potassium acetate) were used to induce *Saccharomyces* sporulation. Selective dropout medium CSM-U (Bio101, Vista, CA) was prepared with 0.17% yeast nitrogen base without ammonium sulfate or amino acids, 0.1% glutamic acid, 2% glycerol, 0.2% glucose and 200 µg/mL geneticin (G-418) (+GG+G418) for selection of respiratory proficient zygotes. Lactate medium (2% lactate, 0.3% yeast extract, 0.05% NaCl, 0.06% CaCl₂, 0.1% KH₂PO₄, 0.1% NH₄Cl, 0.0003% FeCl₃, with or without 0.1% glucose) [19] was used to select for respiratory proficiency. YPDS (YPD supplemented with 1 M sorbitol) was used for recovery of *Pichia* after transformation, and YPDSZ (YPDS supplemented with 1 mg/mL Zeocin) was used for selection. *Pichia* was grown in BMGY medium and methanol induction was performed in BMMY medium supplemented with 0.05% methanol daily [20,21].

Nucleic acids

S. cerevisiae genomic DNA was isolated from INVSc1 (Invitrogen, Grand Island, NY) using the Qiagen Genomic Tip-100 Kit. The RIL vector directing expression of the rare tRNAs encoded by *argU* (AGA, AGG), *ileY* (AUA), and *leuW* (CUA) was isolated from the BL21-CodonPlus-RIL strain (Stratagene, La Jolla, CA) following spectinomycin amplification, using the Qiagen Midi-Prep Kit.

Modification of pET23a—The pET23a expression vector (Promega, Madison, WI) was modified to remove the unique *Xho*I restriction site. The vector was digested with *Xho*I, polished with T4 DNA polymerase, phosphorylated with T4 polynucleotide kinase and ligated with T4 DNA ligase. The sequence of the modified multilinker region of the vector was verified by direct sequence analysis (DNA Sequencing Core, Oregon Health and Science University).

TwinStrep affinity tag—The TwinStrep affinity tag [22,23] for production of fusion protein with a C-terminal GSSWSHPQFEKGGGSGGGSGGGWSHPQFEKGA was constructed from four complementary and overlapping synthetic oligonucleotides (TwinStrep-1,2,3, and 4) (Table S1). The four oligonucleotides (1 nmol each) were combined in Annealing Buffer (10 mM Tris HCl, pH 8, 1 mM EDTA), denatured at 90 °C, and annealed by slow cooling to room temperature (0.4 °C/min). The reaction mixture was purified (Qiagen PCR Spin Prep column) digested with *Xho*I and *Xba*I and ligated to similarly digested pBAD2 vector [24]. After the insert was verified by direct sequence analysis, the pBAD2-TwinStrep vector was digested with *Nde*I, *Xba*I and *Dra*I restriction endonucleases, and the fragments ligated into *Nde*I/*Nhe*I-cut pET23Δ*Xho*I. (*Dra*I blunt cuts the source vector within the Amp^r selection marker to reduce background.) The identity of the TwinStrep-containing product (pET23TwinStrep) was verified by direct sequence analysis.

EGFP cloning—The coding sequence for enhanced green fluorescent protein (EGFP) was amplified from the pEGFP-C3 vector (Clontech Laboratories, Mountain View, CA) with the PCR primers EGFP-1 and EGFP-2 using Pfu polymerase (Stratagene, LaJolla, CA). The 800 bp product was digested with *Nde*I and *Xba*I. Complementary synthetic oligonucleotides encoding the ScFv [(Gly₄Ser)₃] protein linker (ScFv-1,2) (Table S1) were annealed as described above and digested with *Bgl*II and *Nde*I.

ygr257c cloning—The ygr257c (mtm1) gene (GenBank accession No. Z73042.1) was amplified from INVSc1 genomic template using primers Mtm1 *Hind*III and Mtm1 ScFv, digested (*Hind*III/*Bam*H I) and ligated with annealed, digested ScFv (*Bgl*II/*Nde*I) and EGFP

(*NdeI/XbaI*) to form pYES2mtm1EGFP. *Ygr257c* together with its 5' and 3' flanking regions was amplified from INVSc1 genomic template using primers Pmtm1-A and -B using OneTaq polymerase (New England Bio Labs, Beverly, MA). The PCR product was used as template for a second PCR reaction using the primers Pmtm1-1 and Mtm1 TwinStrep. The 1400 bp product was digested (*AgeI/XhoI*) and ligated together with annealed and digested (*XhoI/XbaI*) TwinStrep oligos into pYES2 (*AgeI/XbaI*) to form pYEPmtm1TwinStrep for expression of TwinStrep tagged YGR257Cp under its native promoter. pYES2mtm1EGFP was digested (*AgeI/XbaI*) dephosphorylated (antartic phosphatase, New England Bio Labs) and the vector arms ligated with similarly digested Pmtm1-1/Mtm1 TwinStrep to form pYEPmtm1EGFP. The PCR product from amplification of *ygr257c* using Mtm1 *KpnI* and Mtm1 ScFv or TwinStrep primers was digested (*KpnI*-HF/*Bam*HI and *KpnI*-HF/*Xho*I, respectively) and ligated with pPICZB (*KpnI*-HF/*XbaI*) together with ScFv and EGFP, or TwinStrep sequences to form pPICZmtm1EGFP and pPICZmtm1TwinStrep. The vectors were linearized by *PmeI* digestion for transformation of *P. pastoris* X33. Vectors for bacterial expression were prepared as follows: The 1100 bp *ygr257c* coding sequence was amplified from INVSc1 genomic template using primers Mtm1-1 and Mtm1-2 or Mtm1-3, and digested (*NdeI/XhoI*) for ligation with similarly digested pET23 (for production of WT protein in *E. coli*) or pET23TwinStrep. The PCR product was digested (*NdeI/Bsp*HI) for ligation with the 3' end of mtm1-EGFP fusion coding sequence (from *Bsp*HI/*XbaI*/*Dra*I digestion of pYES2mtm1EGFP) into the *NdeI/NheI*-cut vector arms of pET23Δ*XhoI* to form pET23mtm1EGFP (for production of C-terminal EGFP fusion protein). The sequences of the entire inserts of each of the products were verified by nucleotide sequence analysis.

QuikChange mutagenesis—Silent mutagenesis of the coding sequence was performed to replace Leu TTG codons with TTA or CTG codons using a 5'-phosphorylated mutagenic oligonucleotide (L78QC, L100QC, or L121/L127QC) (Table S1) in combination with the expression vector (pET23mtm1, pET23mtm1TwinStrep or pET23mtm1EGFP) in a QuikChange Multi II (Stratagene, La Jolla, CA) reaction performed according to the manufacturer's instructions. The products were digested with *DpnI* and transformed into XL-2 Blue for selection and amplification. The sequences of the entire coding regions of each of the products were verified by nucleotide sequence analysis.

Biological methods

Preparation of electrocompetent cells—*S. cerevisiae* was grown in YPD medium overnight at 30 °C with shaking, washed three times with ice cold sterile water, once with cold 1 M sorbitol, and resuspended in 0.5 mL of 1 M sorbitol. Electrocompetent *P. pastoris* was similarly prepared, but incubated for 15 min in 100 mL fresh YPD medium containing 25 mM DTT and 25 mM HEPES pH 8 before washing with ice cold sterile water. *E. coli* cultures were inoculated into YPD with appropriate selection, grown overnight at 37 °C with shaking, centrifuged, washed three times with ice cold sterile water, resuspended in 10% glycerol, centrifuged, and resuspended in 0.5 mL 10% glycerol.

Electrotransformation—Electrotransformation of electrocompetent cells was performed using an Eppendorf Electroporator 2510 (Eppendorf, Hamburg, Germany). Bacterial transformation was performed in a BTX 1 mm gap electroporation cuvette at 1.8×10^3 V/cm. Following transformation, 0.5 mL SOC medium was added and the cells incubated 1 h at 37 °C for recovery before spreading on selection medium. Yeast transformation was performed in a BTX 0.2 mm gap electroporation cuvette at 1×10^4 V/cm. Following transformation, 0.5 mL YPDS medium was added and the cells were incubated 1 h at 30 °C for recovery before spreading on selection medium.

Mating cross to restore respiratory proficiency—*S. cerevisiae* BY4741 MATa ygr257c::KanMX | pYEPmtm1TwinStrep (rho-minus) and *S. cerevisiae* BY4739 MATalpha (rho-plus) haploid strains were grown in YPD medium to OD_{600nm} = 1. Equal volumes (1 mL) of the two cultures were mixed, vortexed 3× briefly and 1× for 10 s, spotted in 40 µL drops onto CSM-U+ Glucose agar and incubated at room temperature for 2.5 h. Cells resuspended from the mating spots were streaked onto CSM-U+GG medium containing G-418 to select for respiratory-proficient zygotes containing the pYEPmtm1TwinStrep expression vector. A zygote from this cross selected for sporulation [25] was inoculated into 25 mL PSP2 presporulation medium for 38 h at 30 °C with shaking. The culture was centrifuged in sterile Oak Ridge tubes, the pellet washed with sterile deionized water, suspended in 20 mL 1% KAc sporulation medium and incubated at 30 °C with shaking. For random spore analysis, a 5 day old 1% KAc culture (~25% tetrads) was centrifuged and resuspended in 2 mL sterile deionized water. An aliquot (0.5 mL) was diluted into 5 mL of sterile deionized H₂O containing 15 mM mercaptoethanol and 0.75 mg Zymolyase 100-T (Seikagaku Corporation, Tokyo, Japan) and left overnight at 4 °C. To disrupt the tetrads, 5 mL 1.5% NP-40 was added to the suspension and the mixture was sonicated at 65% maximum power (30 s, 3×) using a Branson Digital Sonifier 250 (Branson Ultrasonics, Laredo, TX) equipped with a microtip. An aliquot was spread onto CSM-U+GG+G-418 selection agar and incubated at 30 °C for two days. Single colonies were cleaned by re-streaking and haploid daughter clones were isolated for PCR mating type analysis [26]. A clone verified as MATa haploid progeny was grown on lactate medium to select for respiratory proficiency and mitochondrial function.

Protein purification

Inclusion body prep—Inclusion bodies were purified from *E. coli* BL21 Star (DE3) | RIL | pET23mtm1TwinStrep(L78QC). A fresh ZYPG plate was used to inoculate four 2 L baffled flasks each containing 500 mL of ZYP-5052 induction medium supplemented with carbenicillin and chloramphenicol. Cultures were incubated with shaking at 30 °C for 26–30 h. Cell mass (typically 15–25 g) was collected by centrifugation, washed with 500 mL of PBS (150 mM NaCl, 10 mM Na₂HPO₄ buffer pH 7.2) and stored at –80 °C. Cells were thawed and suspended in 40 mL of 50 mM Tris–HCl pH 8 containing 1 mM each of PMSF, DTT and EDTA and broken by sonication using a VibraCell (Sonics & Materials, Danbury, CT) equipped with a 3/4" Ti tip, maintaining the temperature below 10 °C with an ice–salt slurry. The pellet from centrifugation (25,000×g, 30 min, 4 °C) was resuspended by sonication with a model FS-14 solid state ultrasonic bath (Fisher Scientific, Pittsburgh, PA) in the same buffer without EDTA and with the addition of 0.5 mM CaCl₂, 10 mM MgCl₂, 40 mg lysozyme and 1 mg DNase I and the mixture incubated on ice for 1 h. The inclusion body material was separated from digested cell wall by centrifugation at 22,000×g for 30 min and further purified by repeated sonication and centrifugation, first with 40 mL of 10% B-Per (Pierce Biotechnology, Rockford, IL) in 50 mM Tris–HCl buffer pH 8 containing 1 mM each of PMSF, DTT and EDTA (3×); then with 2% deoxycholate (DOC) in the same buffer (3×); and finally the pellet was washed in the same buffer without detergent (2×).

Solubilization and refolding of YGR257Cp inclusion body—Inclusion body material containing YGR257Cp was solubilized and refolded by two separate procedures. For high pH solubilization [27], 200 mg of wet purified inclusion body pellet was suspended in 1 mL of deionized water and added to 9 mL of freshly prepared solubilization solution (final concentration: 2 M urea, 100 mM Tris–HCl pH 8.5, 1 mM DTT, 0.1 % Brij-35 containing 75 mM NaOH). The mixture was centrifuged (14,000×g, 10 min) and the clear supernatant added dropwise with gentle swirling to 100 mL of freshly prepared ice cold refolding solution (50 mM Tris–HCl pH 8.5, 5% sucrose, 2 M urea, 5 mM cysteamine, 2.5 mM cystamine and 0.1% Brij-35). The addition was completed in 15 min and the solution

was incubated on ice for a further 30 min. Immediately thereafter, the clear solution was concentrated to 3 mL by ultrafiltration over a YM-10 membrane and the urea removed by desalting over BioGel P-30 (2.5 × 15 cm) equilibrated with 50 mM Tris-HCl pH 8 containing 1 mM DTT and 0.1% Brij-35. Refolded Mtm1p-TwinStrep tag fusion protein (in 50 mM Na₂HPO₄ pH 8, 300 mM NaCl, 0.5 mM DTT, 0.1% Brij-35) was further purified by affinity chromatography over Strep-Tactin Superflow resin (Qiagen, Valencia, CA) according to the manufacturer's instructions.

For detergent solubilization and refolding [4], 200 mg of wet inclusion body pellet was suspended in a mixture of 1 mL of 2.5% sarkosyl (sodium *N*-lauroyl sarcosinate) in 10 mM Tris-HCl pH 8, 0.1 mM EDTA and 1 mM DTT and dispersed in an ultrasonic bath for 5 min at room temperature. The solution was centrifuged (14,000×*g*, 30 min) and the clear supernatant was diluted 25-fold (to 0.1% sarkosyl) with 50 mM NaHPO₄ buffer pH 8 containing 300 mM NaCl and 0.5 mM DTT. TwinStrep tagged protein was further purified by Strep-Tactin affinity chromatography as described above with either 0.1% Sarkosyl or 0.1% Brij-35 in the elution buffer.

Analytical methods

Fluorescence spectroscopy—EGFP fluorescence in bacteria and *Pichia* whole cells, and fluorescence spectra of purified protein samples were measured using a Cary Eclipse spectrofluorometer (Varian Instruments, Walnut Creek, CA) equipped with automatic temperature control. Cells were pelleted, washed and resuspended in PBS (50 mM sodium phosphate buffer pH 7 containing 200 mM sodium chloride), and diluted to OD_{600nm} ~0.1 for fluorescence measurements. For detection of membrane-localized EGFP fusion protein, membranes were purified from bacterial cell-free extract by ultracentrifugation as previously described [28]. EGFP was excited at 490 nm and fluorescence emission detected at 510 nm. Intrinsic protein fluorescence of purified YGR257Cp was excited at 290 nm after dilution of the refolded protein into 10 mM MOPS pH 7 containing 0.5 mM tris (2-carboxyethyl) phosphine (TCEP) and 0.1% Brij-35 at 25 °C in a quartz cuvette. Kinetic timecourses were recorded after each addition of titrant in order to monitor the progress of binding during the titration. Titration data was analyzed using a model incorporating both saturation binding and solution quenching terms Eq. (1):

$$F = F_0(1 - (Q_L \times c / (K_D + c)) / (1 + K_{SV}c)) \quad (1)$$

where F is the observed fluorescence intensity, F_0 is the unperturbed initial fluorescence intensity, Q_L is the fractional quenching resulting from ligand binding at saturation, K_D is the ligand dissociation constant, c is the ligand concentration, and K_{SV} the Stern-Volmer constant [29].

Protein characterization—Protein concentration of purified YGR257Cp protein was determined in the presence of 0.1% Brij-35 detergent using bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL). The optical absorption density at 280 nm (OD_{280nm}) was also routinely recorded for the purified proteins. The combined BCA assay results and OD_{280nm} absorption measurements were used to evaluate the purity of the protein by comparing the observed mass extinction coefficient ($E_{280nm}^{0.1\%}$) with the predicted value obtained from the ExPASy ProtParam tool [30] ($E_{280nm}^{0.1\%} = 1.65$). Total cellular protein was prepared by the quantitative proteomics method, as previously described [31,32]. Proteins resolved by SDS-PAGE (Bio Rad Ready-Gel) were stained with Pierce GelCode Blue Safe protein staining solution (Thermo Scientific). Gels were digitized using a scanner and analyzed with the NIH Image-J densitometry software tool [33]. Lumi-light western blotting kit (Roche Applied Science, Indianapolis, IN) and mouse anti-Strep-tag II

monoclonal antibody (IBA BioTAGnology, Olivette, MO) were used to detect TwinStrep tagged YGR257Cp fusion protein. Quantitation of free sulfhydryl groups in folded YGR257Cp was performed using the 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) assay on protein in guanidinium hydrochloride as described previously [34,35]. Dithiothreitol was removed from the protein immediately before the DTNB test using a Bio Gel P-30 desalting column (1 × 19 cm) equilibrated with 50 mM Tris-HCl pH 8 containing 0.1% Brij-35.

Ultraviolet circular dichroism—Concentrated protein samples from both refolding procedures were prepared for UV CD experiments by exchanging into a low UV absorption buffer (10 mM KH₂PO₄, 10 mM NaCl, 0.1% Brij-35 pH 7) by desalting (BioGel P-30, 1 × 20 cm). Data was collected on samples in a 1:10 dilution of the sample buffer to a final protein concentration of 110 µg/mL in a 1 mm pathlength quartz CD cell. Spectra were acquired using an AVIV Model 41MCD circular dichroism spectrometer (AVIV Associates, Lakewood, NJ) with a high pressure xenon arc UV source. The experimental spectra were submitted to the DICHROWEB web server [36,37] for analysis.

Phyre2 protein structure prediction—The predicted YGR257Cp polypeptide sequence was submitted to the Phyre2 Protein Fold Recognition Server [38] and the homology model structure retrieved for analysis. Since the threading algorithm deleted residues 50–96 during optimization, that sequence was independently submitted for analysis, and the structures with the highest confidence scores were joined at the sequence boundaries using the structure editing tools of Chimera [39]. The helical content of the predicted structure was evaluated from the HELIX secondary structure assignment of residues in the Phyre2 coordinate file.

Results and discussion

YGR257Cp is essential for maintaining healthy mitochondria in yeast. Deletion of the *ygr257c* genomic locus results in irreversible loss of mitochondrial functions (cytoplasmic petite, rho-minus respiratory phenotype) [9,40] that cannot be repaired by simply expressing vector-encoded YGR257Cp in *S. cerevisiae* *ygr257c::KanMX*. However, respiratory proficiency can be restored through cytoplasmic inheritance of healthy mitochondria in haploid daughter cells arising from a mating cross with a rho-plus strain [41,42] (Fig. 1 top), creating a rho-plus derivative of *S. cerevisiae* *ygr257c::KanMX* that may be useful for *in vivo* functional studies of YGR257Cp. In the construction of the rho-plus daughter strain, nutritional and antibiotic selection was used to screen progeny (-U for retention of the pYEPmtm1 plasmid; GG for respiratory proficiency; G418 for *ygr257c::kanMX* gene replacement) and mating type PCR [26] was used to verify haploidy and to assign mating type. The results (Fig. 1, top) show that vector-encoded YGR257Cp-TwinStrep fusion supports full functional complementation of the genetic defect in *S. cerevisiae* *ygr257c::KanMX*, demonstrating that the affinity tag does not interfere with the function of the protein *in vivo*. However, very low expression level is observed in *Saccharomyces* when YGR257Cp is expressed under its native promoter (Fig. 1, bottom, lane 2), and it is only detected by overexposure.

P. pastoris is well known as a microbial cell factory for recombinant production of eukaryotic proteins [20,43]. YGR257Cp was expressed in *P. pastoris* either as a C-terminal EGFP or as TwinStrep tag [22,23] fusion construct in an attempt to achieve higher yields of the protein. Fluorescence was detected in washed cell suspensions from cultures expressing the YGR257Cp-EGFP fusion, increasing over five days of methanol induction and reaching a maximum after 3 days (data not shown). Western blot analysis of the total proteomics lysate shows that YGR257Cp-TwinStrep fusion protein expressed in *P. pastoris* is heterogeneous, with one band consistent with the predicted mass (43977.6 kDa) and

multiple bands at higher mass (Fig. 1, bottom, lane 3). In *Pichia*, high level expression of mitochondrial membrane proteins may lead to mistargeting to secretory membranes where glycosylation can occur [44], possibly accounting for the higher mass species observed in the western blot. Six N-X-S/T motifs are present in the protein sequence (at N5, N97, N103, N207, N302 and N310) that could potentially serve as N-linked glycosylation sites, and the structure of YGR257Cp predicted by Phyre2 homology threading (Fig. 2) suggests that N97, N103, N302 and N310 will all be located on the luminal side of the golgi membrane, where they could be N-glycosylated, if the protein inserts from the cytosol. *P. pastoris* clearly provides significant advantages over *Saccharomyces* for the production of YGR257Cp, and the heterogeneity may not affect functional studies. However, isolation from purified mitochondria may be required to recover the homogenous protein.

In order to avoid protein heterogeneity, we have investigated prokaryotic expression in T7-promoter based systems based on Studier autoinduction [16] of YGR257Cp in the pET23 expression vector in *E. coli* BL21Star (DE3), a host with enhanced mRNA stability as a result of a mutation in RNase E endonuclease that interferes with mRNA degradation [45,46] (Fig. 3A, lane 1). The relatively low expression level suggested codon bias as a possible limiting factor. The codon adaptation index (CAI) [47] for *E. coli* expression of the yeast gene is reasonably high (0.606), but the presence of several rare codons (12 AGG and AGA (Arg); 14 ATA (Ile); 3 CTA (Leu)) including three tandem occurrences, led us to explore the effect of supplementing these rare tRNAs by co-transformation of the BL21 Star (DE3) host with RIL plasmid DNA previously isolated from the CodonPlus expression host (Fig. 3A, lane 2). By mobilizing the RIL plasmid into different genomic backgrounds, it is possible to combine the expression-enhancing effects of rare tRNA supplementation with other features, creating novel expression hosts. An increase in expression level is observed in the presence of the RIL plasmid (Fig. 3A, lanes 1–4), demonstrating that codon bias is a limiting factor for expression of YGR257Cp in *E. coli*.

Three host strains (BL21(DE3), C41(DE3) and BL21 Star (DE3), with and without RIL rare tRNA supplementation) were investigated for optimization of expression yield by Studier autoinduction at two temperatures. The highest expression levels occurred at 30 °C (Fig. 4, bottom) after an extended induction period although significant expression was also observed at 37 °C (Fig. 4, top). Relatively low level YGR257Cp expression was detected in BL21(DE3) and C41(DE3) without RIL grown at 30 °C by western blot analysis (Fig. 4, lanes 1 and 2). In contrast, expression is clearly observed in BL21 Star (DE3) even without RIL, and increases further with RIL rare tRNA supplementation (Fig. 4, lane 3 (±RIL)).

While it is not obvious in the GelCode Blue-stained gel (Fig. 3A, lane 3), an N-terminal truncation product (estimated mass, 37 kDa) is detected by western blotting of TwinStrep tagged YGR257Cp expressed in either BL21 Star (DE3) or the Walker strain, C41 (DE3), using anti-StrepTag MAB (Fig. 3C, lane 3, Fig. 5, lane 1). The relative yields of full-length and truncated protein products appear to be sensitive to both host strain and expression conditions, but the truncated form was always present under conditions that led to the highest overall protein production (Fig. 4). Attempts to obtain an exact mass of this fragment by ESI mass spectrometry were unsuccessful, probably because of poor ionization of the protein. However, the lack of corresponding N-terminal fragments in the GelCode Blue-stained gel argues against proteolytic cleavage as the source of the truncated protein, suggesting the possibility that N-terminal truncation might result from secondary translation initiation. Eukaryotic and prokaryotic ribosomes have distinct mechanisms for initiation, and, as a result, internal (intracistronic) initiation sites may be present in eukaryotic coding sequences when expressed in a prokaryotic host. Once identified, these sites can be eliminated by silent mutagenesis, replacing the initiation codon with a synonymous codon that will not function as an initiation signal. In *E. coli*, translation initiation factor IF3

restricts initiation to the three canonical mRNA codons AUG (Met), GUG (Val) and UUG (Leu), with AUG being the preferred initiation site [48]. Scanning the *ygr257c* coding sequence did not reveal any putative Met initiation sites consistent with the observed mass, but several putative Leu initiation sites (Leu78, Leu100, L121, L127) were identified that would generate truncation products in the observed mass range (Table 1). Site-directed silent mutagenesis of these candidates, substituting a synonymous Leu codon (TTA or CTG) for the putative initiation codon (TTG), allowed us to identify Leu78 as the secondary intracistronic initiation site (Fig. 5, lane 2). It is not clear what makes initiation at Leu78 especially favorable, but the presence of an amber stop codon 17 nt upstream of the potential Shine–Delgarno sequence (Table 1) may facilitate internal ribosomal entry [49], particularly in stationary phase, and factors such as mRNA secondary structure may also play a role in determining the initiation efficiency. Out-of-frame initiation may also occur, but would not be detected in these experiments. Alternate translation initiation is likely to be a common problem for expression of eukaryotic genes in bacteria, but may not be obvious at lower expression levels, or in the absence of epitope tags that allow immunological detection. For YGR257Cp, eliminating the secondary translation initiation site increased the yield of the full length protein nearly two-fold (Fig. 3B, lanes 3 and 4), indicating that this type of secondary intracistronic initiation can have a significant effect on protein yield.

Recombinant YGR257p forms inclusion bodies under the expression conditions investigated above. In an attempt to produce native, folded and membrane-localized YGR257Cp in *E. coli*, we explored using the Walker strain [15] C41(DE3), originally developed to facilitate membrane-localized expression of recombinant proteins [50], for expressing a YGR257Cp EGFP fusion protein, using EGFP fluorescence to monitor formation of the native folded product [28,51]. At low temperature (20 °C) an intense fluorescence signal grew over 3 days (data not shown), indicating expression of folded EGFP product, but the cell lysate contained free EGFP domain rather than the YGR257Cp fusion construct. Also, membranes purified from the broken cells [28] lacked significant fluorescence, indicating the absence of membrane-localized YGR257Cp-EGFP fusion product. It seems likely that expression of the isolated EGFP domain instead of the full-length fusion product, in spite of the lack of an L78 initiation site in the expression cassette, is the result of additional intracistronic translation initiation sites near the 3' end of *ygr257c* (Table 1).

The BL21 Star (DE3) RIL expression host was selected for production of recombinant YGR257Cp as inclusion bodies by autoinduction at 30 °C. After breaking cells by sonication, YGR257Cp, representing nearly 30% of the total cellular protein (Fig. 6, lane 1), was present in the cell-free pellet (Fig. 6, lane 2). After digestion of cell wall material and DNA, the pellet (Fig. 6, lane 3) was subjected to repeated sonication and washing with 10% B-Per and 2% DOC (Fig. 6, lanes 4 and 5). SDS–PAGE gel densitometry analysis showed that after the final wash the protein was ~80% pure (Fig. 6B, lane 5). The insolubility of the protein at the early stages of this procedure presents a challenge to quantitative description of the purification process, but the basic features of the purification protocol are summarized in Table 2. Similar expression and purification results were obtained for untagged YGR257Cp (data not shown).

Detergent-washed inclusion bodies were used for protein refolding studies. Two published refolding procedures (involving high pH [27] or Sarkosyl [4] solubilization) were applied to the inclusion bodies. The high pH solubilization procedure was modified for membrane proteins by refolding in the presence of detergent (Brij-35). The protein was refolded by lowering the pH and by dilution with refolding buffer containing a thiol/disulfide (cysteamine/cystamine) redox buffer, 2 M urea and 0.1% Brij-35. After refolding, the urea was immediately removed to avoid protein carbamylation [52]. Attempts to simplify the refolding process by eliminating urea or replacing 0.1% Brij-35 with *n*-dodecyl β -maltoside

were unsuccessful. On the other hand, treatment with Sarkosyl results in single-step solubilization and refolding and therefore has significant advantages for refolding YGR257Cp, but may not be generally applicable to other membrane proteins.

The refolded YGR257CpTwinStrep was further purified by Strep-Tactin affinity chromatography in buffer containing 0.1% Sarkosyl or 0.1% Brij-35, with approximately 40% yield (Table 2). Higher Sarkosyl concentrations interfere with the performance of the Strep-Tactin affinity resin, and when the Strep-Tactin column was equilibrated in buffer containing Sarkosyl at the critical micelle concentration (CMC) concentration (~0.5%) the yield of YGR257CpTwinStrep was reduced to less than 10%. Refolded YGR257Cp remains in solution in buffer containing Sarkosyl below the CMC (0.1%), although the protein appears to be aggregated since it elutes from a Sephacryl S-200-HR size exclusion chromatography column as a single peak in the void volume. (This type of aggregation is often observed for integral membrane proteins in detergent below the CMC [53].) After column purification, the ($E_{280\text{nm}}^{0.1\%}$) evaluated from the combination of BCA protein quantitation and OD_{280nm} increases from 1.06 to 1.64, close to the predicted value ($E_{280\text{nm}}^{0.1\%}=1.65$).

The UV CD spectra of proteins prepared by both refolding procedures (Fig. 7) demonstrate that a significant fraction of α -helical secondary structure is present in solubilized recombinant YGR257Cp. The α -helical content of the protein is estimated to be 27% based on quantitative analysis of the UV CD spectra [36,37], compared to approximately 50% α -helical content predicted by the Phyre2 fold (Fig. 2). However, the confidence score for the Phyre2 analysis of the matrix domain is very low, and the predicted structure includes several proline residues within α -helical segments, which is inconsistent with the helix-breaking character of that residue. Thus, the Phyre2 prediction of helical content may be considered an upper limit. Thiol quantitation by DTNB analysis shows that there are four to five (4.3 ± 0.1) free sulfhydryl groups in both protein samples, consistent with the Phyre2 homology structural prediction of five free sulfhydryls and two disulfide linkages based on the proximity of the side chains (Fig. 2).

Correct folding of YGR257Cp should permit carrier substrate binding. Although previous genetic and phenotypic studies have not succeeded in identifying a substrate for this carrier protein, they do provide some clues. In particular, ygr257c knockout yeast exhibit striking defects in mitochondrial iron homeostasis, including disruption of iron-sulfur cluster assembly [10] and a block in the initial step of heme biosynthesis [12]. Because key enzymes in both iron sulfur cluster and heme biosynthetic pathways (cysteine desulfurase, δ -aminolevulinate synthase) are pyridoxal 5'-phosphate (PLP)-dependent enzymes, we were led to consider PLP as a possible substrate for YGR257Cp. The availability of purified YGR257Cp has allowed us to test this novel hypothesis, titrating the refolded recombinant protein with PLP (Fig. 8). The intrinsic tryptophan luminescence of the protein is strongly quenched in the presence of pyridoxal 5'-phosphate, reflecting high affinity binding ($K_D = 0.7 \pm 0.02 \mu\text{M}$), together with solution quenching extending over the higher concentration range ($K_{SV} = 9 \pm 3 \times 10^3 \text{ M}^{-1}$). These results show that YGR257Cp binds PLP with high affinity, supporting a new assignment of the function of this mitochondrial carrier as a high-affinity PLP transporter.

In conclusion, we have developed an efficient method for expression, purification and refolding of the recombinant YGR257Cp yeast mitochondrial carrier protein in *E. coli*, which will facilitate further structural and functional characterization of the pure protein. The availability of the purified recombinant protein has allowed us to test a new hypothesis for the biological function of YGR257Cp, supporting its assignment as a high affinity mitochondrial pyridoxal 5'-phosphate carrier. This is the first evidence for an essential

mitochondrial PLP transport function in eukaryotes. Further understanding the biochemical function of this important yeast solute carrier will provide important insight into the role of human homologs in health and disease [54,55].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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.pep.2013.10.014>.

Fig. 1.

Expression of TwinStrep tagged YGR257Cp in *Saccharomyces cerevisiae* and *Pichia pastoris*. (Top) Growth curves for *S. cerevisiae* strains on lactate medium. (a, triangles) BY4741 ygr257c::KanMX | pYES2; (b, circles) BY4700 | pYES2; (c, squares) BY4741 ygr257c::KanMX | pYEPmtm1TwinStrep. A smooth curve is drawn through the data to guide the eye. (Bottom) Western blot analysis of (M) Strep-tag protein ladder; (1) YGR257CpTwinStrep purified from *E. coli*; (2 and 3) quantitative proteomics lysates prepared from (2) *S. cerevisiae* BY4741 ygr257c::KanMX | pYEPmtm1TwinStrep or (3) *P. pastoris* X33 containing the PICZmtm1TwinStrep expression cassette.

Fig. 2. Homology model structure for YGR257Cp. Stereoview of the structure predicted by Phyre2 homology modeling algorithm [38] and visualized with Chimera [39]. Cysteine side chains are rendered as yellow van der Waals spheres. IMS, intermembrane space; IMM, inner mitochondrial membrane; Matrix, mitochondrial matrix. The membrane topology of the carrier protein is based on analogy with the ADP/ATP exchanger (PDB ID 1okc). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 3.

Effects of rare tRNA supplementation on YGR257Cp expression in *E. coli*. (A) GelCode Blue stained SDS-PAGE gel. M, low range MW protein standards; BL21 Star (DE3) expression cultures: (G), glucose-repressed control; (1–4) following Studier autoinduction at 30 °C for 26 h, (1) WT YGR257Cp, –RIL; (2) YGR257Cp L78QC, –RIL; (3) WT YGR257Cp, +RIL; (4) YGR257Cp L78QC, +RIL. (B) Densitometry analysis of gel data. (C) Same experiment as (A) for *E. coli* BL21 Star (DE3) host expressing TwinStrep tagged YGR257Cp for Western blot analysis. M, Strep-tag protein ladder.

Fig. 4. Optimization of TwinStrep tagged YGR257Cp production in *E. coli* expression hosts. (M) low range MW protein standards; (C) glucose-repressed control; (1) BL21(DE3); (2) C41(DE3); (3) BL21 Star (DE3) containing pET23mtm1TwinStrep after autoinduction for 42 h at (top) 37 or (bottom) 30 °C.

Fig. 5. Identification of a secondary intracistronic translation initiation site in the *ygr257c* coding sequence. Western blot analysis of expression products from *E. coli* C41(DE3) host containing pET23 vector encoding TwinStrep tagged: (1) WT YGR257Cp; (2) YGR257Cp L78QC; (3) YGR257Cp L100QC; (4) YGR257Cp L121/L127QC. M, Strep-tag protein ladder.

Fig. 6. Purification of YGR257CpTwinStrep from *E. coli*. (A) GelCode Blue stained SDS-PAGE gel. M, low range MW protein standards; (1) whole cell suspension; (2) cell-free pellet; (3) lysozyme-treated pellet; (4) B-Per washed pellet; (5) DOC-washed pellet. (B) Densitometry analysis of gel data.

Fig. 7. Ultraviolet circular dichroism spectra for refolded YGR257CpTwinStrep. (1, red line) detergent-solubilized protein; (2, blue line) high pH solubilized protein in 1mM KH_2PO_4 buffer pH 7 containing 1 mM NaCl and 0.01% Brij-35. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 8. Fluorescence-detected binding of pyridoxal 5'-phosphate to refolded recombinant YGR257Cp. Purified, refolded protein was diluted into 10 mM MOPS pH 7 containing 0.5 mM TCEP and 0.1% Brij-35 for analysis ($\lambda_{\text{EX}} = 290$ nm). Freshly prepared pyridoxal 5'-phosphate was added in 0.33 μM increments, and the fluorescence spectrum recorded following equilibration. The observed fluorescence intensity at 340 nm was fit to Eq. (1) (Materials and methods) to estimate ligand binding affinity ($K_{\text{D}} = 0.7 \pm 0.02$ μM) and the Stern–Volmer quenching constant ($K_{\text{SV}} = 9 \pm 3 \times 10^3$ M^{-1}).

Table 1

Potential in-frame intracistronic translation initiation sites within the *ygr257c* coding sequence.

Putative initiation codon	Sequence context ^a	Predicted mass of N-terminal truncation product ^b (kDa)
<i>1. Met (ATG)</i>		
M16	taa(-12) aaa gaacgaatg	42243.7
M200, M201	aa agacatggatgatg	22355.6, 22224.4
M215	taa(-12) agg aaatgaaaatg	20507.4
<i>2. Leu (TTG)</i>		
L78	tga(-17)ggaggacagaacttg	35836.3
L100	aaaa attcatcgttg	33284.5
L121	ga agggtattacaagtttg	31075.9
L127	ggagg gggtatttcttg	30363.1
L364	tga(-19)ggaacaaattg	3610.7
<i>3. Val (GTG)</i>		
V216	taa(-12) agg aaatgaaaatgggtg	20376.2

^a Potential Shine–Delgarno sequence indicated by bold underline; putative initiation codon shown in bold.

^b Mass predicted for TwinStrep tagged YGR257Cp.

Table 2Purification of recombinant YGR257Cp from *E. coli*.

Purification step	Mass (g)	OD ₂₈₀ ^a	$E_{280\text{nm}}^{0.1\%}$ ^b	Protein yield (%)
1. Wet cell pellet ^c	12			
2. Inclusion body	0.8	180 ^d	1.06	100
3. Strep-Tactin	0.044 ^e	72	1.64	40

^aOptical density at 280 nm × volume (mL).^bExperimental value for comparison with the theoretical $E_{280\text{nm}}^{0.1\%} = 1.65$.^cFrom 1 L of culture medium.^dAfter refolding in 2.5% Sarkosyl.^eFrom BCA protein quantitation.