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## Determination of the Heme Affinity for Yeast Dap1p, and its Importance in Cellular Function†

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### Abstract

Current studies on the *Saccharomyces cerevisiae* protein Dap1p have demonstrated a heme related function within the ergosterol biosynthetic pathway. Here we present data to further the understanding of the role of heme in the proper biological functioning of Dap1p in cellular processes. Firstly, we examined the role of Dap1p in stabilizing the P<sub>450</sub> enzyme, Erg11p, a key protein involved with facilitating ergosterol biosynthesis. Our data indicates that the absence of Dap1p does not affect Erg11p mRNA or protein expression levels, nor the protein degradation rates. Secondly, in order to probe the role of heme in the biological functioning of Dap1p, we measured ferric and ferrous heme binding affinities for Dap1p and the mutant Dap1p<sup>Y138F</sup>, as well as equilibrium midpoint reduction potentials of the Fe(III)/Fe(II) couples. Our results show that both wild-type and mutant proteins bind heme in a 1:1 fashion, possessing tight ferric heme affinities, K<sub>D</sub> values of 400 pM and 200 nM, respectively, but exhibiting weak ferrous affinities, 2 μM and 10 μM respectively. Additionally, the measured reduction potential of Dap1p, which was found to be 307 mV, is similar to that of other mono-tyrosinate hemoproteins. Although the weaker affinity of Dap1p<sup>Y138F</sup> for ferric heme lowers the production of ergosterol with respect to wild-type Dap1p, it is still sufficient to rescue the growth sensitivity of *dap1Δ* to fluconazole and MMS, suggesting that the activity of Dap1p is directly related to its ability to bind heme.

The yeast protein, Dap1p, belongs to a highly conserved and ubiquitous protein family known as membrane associated progesterone receptors, MAPR's. The first characterized member of this family was found in porcine liver tissues and was identified by its ability to bind progesterone(1–3). Homologous members of this family have been identified, via sequence alignments, in all eukaryotes, from protozoans to humans. They share a conserved cytochrome b<sub>5</sub> heme binding motif (4), although, the protein sequences lack the two histidines required for cytochrome b<sub>5</sub> binding of heme. Nevertheless, within the last five years, evidence has been mounting towards a biological role for heme binding with several of the well characterized MAPR's, including Dap1p (5–9).

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Yeast Dap1p was first characterized in a deletion strain, *dap1Δ*, by its sensitivity to methyl methanesulfonate (MMS), a DNA damaging agent, and fluconazole/itraconazole, Erg11p sterol synthesis inhibitors (10). *Dap1Δ* strains were later shown to be rescued from the MMS sensitivity by the presence of hemin in the media, both in growth and sterol production (7). Mallory, et al. also demonstrated that over-expression of Erg11p in *dap1Δ* lowered the sensitivity against itraconazole. Although, *dap1Δ* did not affect mRNA levels of Erg11p, Mallory did report lowered protein levels of Erg11p, suggesting a protein regulatory function for Dap1p, possibly as a heme chaperone (7). It was also reported at this time that a Dap1p fusion protein bound heme, although its heme ligation was unknown. The heme binding of Dap1p was further characterized by our group as a penta-coordinate, ferric heme, with Tyr138 as the axial ligand (6). Recently, Hughes et al, reported that Dap1p bound Erg11p in a 1:1 ratio and that heme binding by Dap1p was important for maintaining sterol production, by comparing the sterol profile of wild-type, *dap1Δ*, and Dap1p<sup>Y138F</sup> strains (8). Hughes et al. also demonstrated that the protein level of the human homologue of Erg11p, Cyp51A1, was not affected by the loss of the human homologue of Dap1p, PGRMC1, supporting their hypothesis that MAPR's activate P<sub>450</sub> cytochromes through a protein-protein interaction and not by chaperoning heme, contrary to past hypotheses (5–7). For these reasons, we set out in this study to further characterize the cellular and biochemical properties of Dap1p in an attempt to understand its role in yeast biology in more detail.

## Methods and Materials

### Source of Materials

Restriction endonucleases were obtained from New England BioLabs (Beverly, MA). All other reagents were reagent grade or better and were used without further purification.

### Plasmids, Strains, and Growth Conditions

The *E. coli* protein expression plasmids for Dap1p and Dap1p mutants were constructed as previously reported (6). Briefly, PCR amplified DAP1 gene from yeast genomic DNA was cloned into the pET28a vector (Novagen) generating the pET28a\_DAP1 plasmid. All mutants were subsequently created by site-directed mutagenesis, using a Quikchange kit (Stratagene), and verified by DNA sequencing.

Yeast vector pRS313\_DAP1 containing the native DAP1 promoter was constructed by PCR amplifying the DAP1 open reading frame along with 340 bps of promoter sequence and 254 bps of 3' UTR, cloned into pCR2.1-TOPO (Invitrogen) and sub-cloned into SpeI/Xho I sites of the low-copy vector pRS313. Dap1p<sup>D91G</sup> was made by site directed mutagenesis on both the pRS313\_DAP1 and pET28a\_DAP1 plasmids. Wild-type (BY4741) and isogenic *dap1Δ* (BY4741-*dap1::KanMX*) were transformed with pRS313, pRS313\_DAP1, or pRS313\_DAP1<sup>Y138F</sup> plasmid and grown on selective media.

*pRS413GAL\_ERG11-HA* plasmid was created as follows: EcoR I (5') / Xho I (3') fragment containing the *ERG11* open reading frame plus 3HA tag right before the stop codon was generated by PCR from genomic DNA of BY4741 strain and cloned into the EcoR I and Xho I sites of *pRS413GAL* vector.

*pRS303\_ERG11-HA* was created by sub-cloning ~1.3 Kb Xba I / Xho I C-terminal fragment of *ERG11* from *pRS413GAL\_ERG11-HA* plasmid into a *pRS303* vector. *pRS303\_ERG11-HA* was linearized by cutting with the unique BspM I and Pflm I within the C-terminal of *ERG11* ORF and transformed into BY4741 wild-type and *dap1Δ* strains to generate strains genomically tagged *ERG11-HA*. The integration was confirmed by PCR. The above *ERG11-*

HA tagged strains were grown overnight, diluted, grown in SD-His medium for 4 hr and then harvested for qRT-PCR and western blotting, respectively.

### Quantitative RT-PCR

Early -log phase cells, treated with or without 25  $\mu$ M hemin were harvested and the total RNA was extracted by Qiagen RNeasy mini Kit. Contaminating DNA was removed from 16  $\mu$ l of RNA extract by the addition of 2  $\mu$ l 10X reaction buffer and 2  $\mu$ l of DNase (Promega RQ1 DNase) and incubating for 30 min at 37°C. Reactions were terminated by adding 2  $\mu$ l of Stop Buffer and heating for 10 min at 65°C. cDNA was synthesized using Applied Biosystem TaqMan reverse transcription kit by the following modified protocol. Each 20  $\mu$ l reaction consisted of: 2  $\mu$ l 10X TaqMan RT Buffer, 4.4  $\mu$ l 25 mM MgCl<sub>2</sub>, 4  $\mu$ l dNTP mix, 1  $\mu$ l Random Hexamers, 0.4  $\mu$ l RNase Inhibitor, 0.6  $\mu$ l Multiscribe Reverse Transcriptase (50 U/ $\mu$ l), 7.6  $\mu$ l DNase-treated RNA sample (1  $\mu$ g total RNA). Reactions were incubated at 25°C for 10 min, 48°C for 30 min, followed by heat inactivation at 95°C for 5 min. Prior to quantitative PCR, cDNAs were diluted 1/4 in nuclease-free water.

The following primers were used for real time PCR analyses:

ERG11(f): 5' TTCCGTCGGTGAAGAAGTCGATTACG 3',

ERG11(r): 5' ACATCTGTGTCTACCACCACCGAAAG 3',

TBP(f): 5' CGGTTTCGTGTGACGTTAAATTCC 3',

TBP(r): 5' GCACAGGGTATATAGCTTCAAAGC 3'

. PCR reactions consisted of 4  $\mu$ l diluted template (cDNA, standard genomic DNA, or water), 20  $\mu$ l 2X SybrGreen Taq Mix (Applied Biosystems), 300 nM each primer, and nuclease-free water to make a final volume of 40  $\mu$ l. PCR was carried out using a MJ Research Opticon 2 in duplicate for triplicate cDNA samples and primer sets. Thermocycle profile was as follows: 95°C for 10 min, 40 cycles of 95°C 30 sec and 60°C 1 min, with a final denaturation cycle to examine the DNA melting curves of PCR products.

The cycle thresholds (C(t)) were calculated for samples and genomic DNA standards. For each transcript, the C(t) value was converted to a nanogram genomic DNA equivalent by comparing the C(t) of an unknown to standard curves prepared from genomic DNA of strain BY4741 (1, 0.1, 0.01, 0.001 ng). Slopes for *ERG11* and *TBP* standard curves ranged from 3.3–4.3 and were linear with R<sup>2</sup> of 0.999. Expression was calculated by normalizing the nanogram value for *ERG11* to the nanogram value of *TBP*.

### Western blotting (determination of protein half-life)

Wild-type and isogenic *dap1 $\Delta$*  yeast strains transformed with *pRS413GAL\_ERG11-HA* were grown overnight in SD-His medium containing 2 % raffinose, inoculated in the same media and grown for 4 h. Galactose (0.5%) was added to the SD-His media (2% raffinose) to induce Erg11p protein expression. After 4 h of induction, cells were centrifuged and resuspended in SD-His containing 2% glucose to stop the induction. Aliquots at 0, 60, 120, and 240 mins, after stopping induction, were removed for immunoblot analysis. Protein extracts, with the same amount of OD<sub>600</sub> units by NaOH/  $\beta$ -mercaptoethanol method, were loaded onto 10 % SDS-PAGE and separated by electrophoresis. After transferring, the blot was incubated with a primary rabbit anti-HA antibody Y11 (Santa Cruz Biotech) and HRP-conjugated secondary goat anti-rabbit IgG (Santa Cruz Biotech.), developed by ECL, and recorded on ECL Hyper film (Amersham). The same blot was subsequently incubated with a primary mouse anti-yeast 3-phosphoglycerate kinase (PGK) monoclonal antibody (Molecular Probes Invitrogen) and a HRP- conjugated secondary goat anti-mouse IgG (Santa Cruz Biotech.) as a loading control (11).

### Growth phenotype in Fluconazole or MMS media

The early log-phase cells ( $OD_{600} = 0.2$ ) were serially diluted 10-fold with water and spotted into selective SD media containing either fluconazole or MMS. The plates were incubated at 30°C for 3 days and photographed.

### Purification of Proteins

Purification and expression of Dap1p and Dap1p<sup>Y138F</sup> was from *E.coli* BL21(DE3) cells and has been previously reported (6). Briefly, single colonies of freshly transformed cells were grown in LB broth containing 25 µg/ml of kanamycin (FisherBiotech) at 37°C and 200 rpm until an  $OD_{600}$  of 0.6 was attained. Cultures were then induced with 0.5 mM IPTG and grown overnight at 20°C and 100 rpm. Cell lysates were precipitated with ammonium sulfate, the 10–40% fraction was passed over a methyl hydrophobic interaction column, and the cleanest protein fractions were passed over a size exclusion column. Fractions of greater than 90% purity, as determined by PAGE, were collected, concentrated, and stored at –80°C in 5% glycerol.

### Circular Dichroism

Purified protein samples, 70–90 µM, were measured in a 0.1 mm cuvette on a AVIV Model 60DS, from 190–240 nm, in 0.5 nm increments. The average of five spectra were integrated and measurements of percent alpha-helicity were based on the absorbance at  $\theta_{222}$  nm and calculated with the equation  $-\theta_{222}/33,000 = \% \text{ alpha-helicity}$  (12).

### Measurement of Protein and Heme Extinction Coefficients

Heme content was measured by both ICP-MS analysis and pyridine hemochrome method (6, 13). Protein concentrations were measured by amino acid analysis, AAA, (UC Davis) and Bradford Assay (BioRad). Myoglobin was used as a standard due to its similarity in size to Dap1p and its heme binding. The protein extinction coefficient was back calculated from amino acid analysis by the summation of all amino acids measured against an internal standard, norleucine; a molar protein concentration could then be compared to the electronic spectra (<http://msf.ucdavis.edu/calculations.html>). The contribution of the heme to the UV-Vis absorption at 278 nm was determined by titration of heme into Dap1p and relating it to the Soret band.

### Heme Reconstitution

A freshly prepared 100 × Hemin stock solution in 0.1M NaOH and 50% dimethyl sulfoxide, was used to add 1.5–2 fold excess heme to Dap1p and Dap1p<sup>Y138F</sup> protein solutions. Protein and heme were left over night to equilibrate, and then washed three times with buffer in 10 kDa centricons for removal of unbound heme. Heme concentration was measured by ICP-MS, and protein concentration by UV-Vis absorption and Bradford.

### Determination of Binding Constants

The heme binding affinity of Dap1p was measured using two competition binding methods. First, direct titration of horse skeletal muscle (HSM) apo-myoglobin into as-isolated, ferric Dap1p, with ~30% heme bound, was used to estimate the ferric heme  $K_D$  value of Dap1p. Singular value decomposition of the UV-Vis spectra coupled with fitting to a competition binding model was used to determine the ferric  $K_D^{\text{comp}}$  value (14). The  $K_D$  value of Dap1p was estimated based on the experimentally derived  $K_D^{\text{comp}}$  value and the  $K_D$  value of HSM myoglobin, estimated to be similar to the 28 fM ferric heme  $K_D$  value of sperm whale myoglobin (calculations and methods outlined in the supplemental data) (15). Due to the kinetic

trapping of ferrous heme by myoglobin and the lack of a measured ferrous heme  $K_D$  value, this could not be used to determine the ferrous  $K_D$  value of Dap1p (16).

In order to determine more accurate ferric and ferrous  $K_D$  values for Dap1p, direct competition titration experiments were performed using a synthetic heme protein maquette,  $[\Delta 7\text{-His}]_2$ , whose ferric and ferrous  $K_D$  values are well-described between pH 5.0 and 9.0. At pH 8.0,  $[\Delta 7\text{-His}]_2$  possesses a ferric heme  $K_D$  value of 160 pM and a ferrous heme  $K_D$  value 40 nM and is kinetically competent for heme transfer experiments (17). In the ferric heme state, apo- $[\Delta 7\text{-His}]_2$  was titrated into a solution of 15  $\mu\text{M}$  Dap1p containing 2.8  $\mu\text{M}$  bound heme which resulted in a shift in the Soret band maximum from 398 nm to 412 nm. Heme transfer was evidenced by the increase in the intensity of the Soret at 412 nm which was monitored after an hour equilibration time.

For ferrous heme equilibrium measurements, apo- $[\Delta 7\text{-His}]_2$  was titrated into a solution of 10.6  $\mu\text{M}$  Dap1p containing 2  $\mu\text{M}$  bound heme under anaerobic conditions and maintained under a reducing redox environment, with the addition of a slight excess of sodium dithionite. Over the hour equilibration time the Soret band shifts from 422 nm to 427 nm, indicative of heme transfer to the synthetic protein. The equilibrium concentrations of holo-Dap1p and holo- $[\Delta 7\text{-His}]_2$  were calculated based on the intensity at 427 nm.

The heme binding stoichiometry and heme affinity of Dap1p<sup>Y138F</sup> was determined by direct titration of ferric or ferrous heme, the latter under reducing, anaerobic conditions with a slight excess of sodium dithionite. The affinity of Dap1p<sup>Y138F</sup> for ferric heme was determined by measuring the increase in absorbance at 398 nm and fitting the data to a 1:1 heme:protein binding equilibrium. The affinity of Dap1p<sup>Y138F</sup> for ferrous heme was determined in an analogous fashion using the increase in absorbance at 422 nm.

### Resonance Raman Spectroscopy

Typical enzyme concentration for the resonance Raman (RR) experiments were ~ 150  $\mu\text{M}$  in 25 mM HEPES (pH 7.4) buffer solution. Reduction to the ferrous state was achieved by adding microliter aliquots of a 10 mM sodium dithionite solution to argon-purged samples in the Raman capillary. The carbonyl complex was formed by direct injection of CO (Airgas) in the Raman capillary through a septum. RR spectra were obtained on a custom McPherson 2061/207 spectrometer (0.67 m with variable gratings) equipped with a Princeton instruments liquid N<sub>2</sub>-cooled CCD detector (LN-1100PB) and Kaiser Optical supernotch filters. A krypton laser (Innova 302, Coherent) and an argon laser (Innova 90C, Coherent) were used for the 413 and 514.5 nm excitations, respectively. Spectra were collected in a 90° geometry on samples at room temperature with a collection time of a few minutes. Frequencies were calibrated relative to indene and CCl<sub>4</sub> and are accurate to  $\pm 1 \text{ cm}^{-1}$ . Sample heating and photochemistry was prevented by keeping the laser power below 5 mW and mounting the samples on reciprocating translation stage. Optical spectra were obtained before and after the RR experiments directly in the Raman capillaries and confirmed the integrity of the samples after laser illumination.

### Determination of Heme Reduction Potential of Dap1p

Chemical redox titrations were performed in an anaerobic cuvette equipped with a platinum working and a calomel reference electrode at 22°C (18). Ambient potentials (measured against the standard hydrogen electrode) were adjusted by addition of aliquots (<1  $\mu\text{L}$ ) of sodium dithionite or potassium ferricyanide. Titrations were performed in 20 mM potassium phosphate, 100 mM KCl, pH 8.0. Electrode-solution mediation was facilitated by the following mediators at 10  $\mu\text{M}$  concentration: methyl viologen, benzyl viologen, anthroquinone-2-sulfonate, anthroquinone-2,6-disulfonate, 2-hydroxy-1,4-naphthoquinone, duroquinone, 5-hydroxy-1,4-naphthoquinone, 1,4-naphthoquinone, phenazine ethosulfate, and phenazine



methosulfate. After equilibration at each potential, the optical spectrum was recorded. Heme reduction was followed by the increase in the Soret-band absorption (422 nm) relative to a baseline wavelength (750 nm). Spectral intensity was plotted against potential and the data were fit to a single Nernst equation with  $n = 1.0$  (fixed).

## Results

### mRNA and Protein Expression

mRNA expression levels of ERG11, measured by quantitative real-time PCR in wild-type and *dap1Δ* strains, were found to be unaffected by the absence of Dap1p, as previously reported (7). Additionally, mRNA expression levels were unaffected by the addition of hemin (Figure 1). Protein levels of genomically tagged Erg1p-HA were measured from wild-type and *dap1Δ* yeast strains, standardized to the house keeping protein, PGK. Contrary to previously published results, Erg1p protein levels were found to be unaffected by the absence of Dap1p, relative to PKG (7) (Figure 1). Erg1p protein degradation levels were monitored by western blot and standardized to PKG protein levels at 0, 1, 2, and 4 hours after the induction/repression. The half-life ( $t_{1/2}$ ) of Erg1p was plotted and calculated to be 40 minutes and 38 minutes in wild-type and *dap1Δ* strains, respectively (Figure 2).

### Sensitivity to Fluconazole and MMS

Dap1p, Dap1p<sup>Y138F</sup>, and Dap1p<sup>D91G</sup> were transformed into wild-type and *dap1Δ* yeast strains and plated on selective media. The *dap1Δ* strain exhibits a growth sensitivity to media containing fluconazole or MMS which can be recovered by native expression of Dap1p but not by Dap1p<sup>D91G</sup> (data not shown), as previously reported by Mallory, et al (7). However, Dap1p<sup>Y138F</sup> can rescue the growth sensitivity for both fluconazole and MMS to wild-type growth, as shown in Figure 3.

### Expression and Purification

Dap1p and Dap1p<sup>Y138F</sup> were both expressed in *E.coli* at high levels, as previously reported (6). Attempts to purify a soluble form of untagged or his-tagged Dap1p<sup>D91G</sup> were unsuccessful due to inclusion bodies, despite high levels of expression.

### Circular Dichroism

Circular dichroism was used to measure percent  $\alpha$ -helicity to insure Dap1p and Dap1p<sup>Y138F</sup> were not grossly misfolded. Dap1p and Dap1p<sup>Y138F</sup> were found to have 48% and 40%  $\alpha$ -helicity respectively, based on  $\theta_{222}$  values of 13,252 and 15,858. These numbers are comparable to the NMR structure of the plant homologue, At2g24940.1 (PDB = 1T0G), which indicates ~31% alpha-helicity. The difference may be accounted for by both the smaller size of At2g24940.1 (101 amino acids compared to 152 for Dap1p) and the lack of bound heme from its *in vitro* expression (19).

### Determination of the Heme Extinction Coefficient

Quantification of heme concentration is typically done by measurement of the ferrous pyridine heme complex (13); however we have found the standard extinction coefficients associated with this method vary with both buffer and pH changes. Instead, we used ICP-MS to determine heme concentration based on iron analysis. From multiple protein preparations, we averaged an extinction coefficient of  $96,800 \pm 6,000 \text{ M}^{-1} \text{ cm}^{-1}$  for Dap1p, which is within error of our previously reported value of  $99,000 \text{ M}^{-1} \text{ cm}^{-1}$  (6). Based on this value, a small feature for Dap1p<sup>Y138F</sup>, as purified, is noted at 399 nm, corresponding to less than 2% heme bound (Figure 4).

## Determination of Protein Extinction Coefficient

The protein extinction coefficient for Dap1p<sup>Y138F</sup> was measured by both amino acid analysis and Bradford assay. The average  $\epsilon_{278}$  for Dap1p<sup>Y138F</sup> (<2% heme bound) was measured as  $17,000 \pm 3,500 \text{ M}^{-1} \text{ cm}^{-1}$ , which is within the range of the predicted extinction coefficient of  $15,500 \text{ M}^{-1} \text{ cm}^{-1}$  (ExPASy ProtParam tool). Determination of the  $\epsilon_{278}$  for wild-type Dap1p is complicated by the additional heme absorption at 278 nm. To account for this variable we assumed the protein extinction coefficient for wild-type to be similar to that of the mutant Dap1p<sup>Y138F</sup> (collaborated with the predicted value of  $17,000 \text{ M}^{-1} \text{ cm}^{-1}$ , ExPASy ProtParam tool) and solved for the additional heme extinction coefficient at 278 nm. Based on a titration of heme into a solution of Dap1p, we compared the change at 399 and 278 nm, and using our known heme extinction coefficient of  $96,800 \pm 6,000 \text{ M}^{-1} \text{ cm}^{-1}$ , have found the heme extinction coefficient at 278 nm to be approximately  $22,500 \pm 3770 \text{ M}^{-1} \text{ cm}^{-1}$ .

## Heme Binding Constants

Removal of heme from Dap1p was hampered by the fact that standard methods of heme extraction denatured the protein (20,21). Therefore, to measure the  $K_D$ , we switched to a competitive binding method using HSM apo-myoglobin and a 30% heme-loaded Dap1p sample. This method was used to determine an initial ferric heme dissociation constant of approximately 100 fM for Dap1p, assuming the HSM myoglobin had the same  $K_D$  value as that of sperm whale myoglobin, *i.e.* 28 fM (Supplemental data, Figure S1) (16).

In order to obtain a more accurate  $K_D$  values in both oxidation states, we utilized the synthetic heme protein maquette,  $[\Delta 7\text{-His}]_2$ , which has well determined  $K_D$  values in both the ferric and ferrous heme states (22). As previously reported,  $[\Delta 7\text{-His}]_2$  has a ferric  $K_D$  value 160 pM and a ferrous heme  $K_D$  value 40 nM at pH 8.0 (17). Upon titration of apo- $[\Delta 7\text{-His}]_2$  to a solution of as-isolated Dap1p, under ferric and ferrous conditions,  $K_{\text{comp}}$  values are measured as  $2.5 \pm 1.0$  and  $35 \pm 15$ , respectively, and with 1:1 heme to protein ratios in both cases. The ferric heme dissociation constant value for Dap1p was then calculated to be  $400 \pm 200 \text{ pM}$ , and the ferrous dissociation constant value is  $2 \pm 1 \text{ }\mu\text{M}$  (Figure 5).

For the mutant Dap1p<sup>Y138F</sup>, direct titration of heme was possible due to the low initial concentration of heme (<2%) from purification and a weaker affinity for heme. A protein to heme binding ratio of 1:1 is observed by direct titration and ferric and ferrous heme dissociation constants for Dap1p<sup>Y138F</sup> were measured as  $200 \pm 100 \text{ nM}$  and  $10 \pm 5 \text{ }\mu\text{M}$ , respectively (Figure 6).

## RR characterization of Dap1p

The high-frequency region of the RR spectra of Dap1p in the ferric, ferrous, and ferrous-CO state are shown in Figure 7. The ferric protein displays fully symmetric porphyrin modes  $\nu_4$  and  $\nu_3$  at  $1373$  and  $1489 \text{ cm}^{-1}$ , respectively. These frequencies are characteristic of a 5-coordinate high-spin ferric heme (23). In porphyrins bound to a proximal histidine, the  $\nu_4$  mode dominates the high-frequency spectra, but in Dap1p, as in other hemoproteins lacking a proximal histidine, the  $\nu_4$  and  $\nu_3$  modes have comparable intensities (24,25). Attempts to confirm the iron(III) ligation to a tyrosine with RR experiments using a 514.5-nm excitation were inconclusive (data not shown). Upon reduction with dithionite, the  $\nu_4$  oxidation-state marker band shifts to  $1360 \text{ cm}^{-1}$  while the  $\nu_3$  and  $\nu_{10}$  at  $1471$  and  $1604 \text{ cm}^{-1}$ , respectively, are indicative of a 5-coordinate high-spin configuration (Figure 7). Binding of CO up-shifts these frequencies ( $\nu_4$  at  $1374$ ,  $\nu_3$  at  $1503$ , and  $\nu_{10}$  at  $1638 \text{ cm}^{-1}$ ) and results in the detection of a  $\nu$  (Fe-CO) at  $533 \text{ cm}^{-1}$  (Figure 8), which is characteristic of heme-carbonyl complexes with a weak or no proximal ligand (24, 26–28).

## Electrochemistry of Dap1p

Figure 9 shows the electrochemistry of wild-type Dap1p as evaluated using UV-Vis spectroelectrochemistry. The change in Soret band absorption upon reduction to ferrous heme was fit to an equilibrium midpoint reduction potential of  $-307$  mV vs. SHE. This value is similar to the reduction potential of bovine catalase  $-260$  mV vs. SHE value of bovine catalase, as well as other mono-tyrosinate heme proteins (29,30). Attempts to measure the reduction potential of *holo*-Dap1p<sup>Y138F</sup> were thwarted by the extremely weak binding of ferrous heme to the mutant protein. However, since the reduction potential of a heme protein is determined by the relative stability of the ferric and ferrous heme states, the measured  $K_D$  values can be used to determine the reduction potential of the Dap1p<sup>Y138F</sup> mutant (31). The 200 nM dissociation constant for ferric Dap1p<sup>Y138F</sup> is 2000-fold weaker than the 400 pM value of ferric *holo*-Dap1p. Using the thermodynamic relationship,  $\Delta G = -RT \ln K_{eq}$ , the 2000-fold change in  $K_D$  represents a  $+4.5$  kcal/mol destabilization of the ferric state Dap1p<sup>Y138F</sup> relative to Dap1p. Since  $\Delta E = \Delta \Delta G/nF$ , the  $+4.5$  kcal/mol destabilization of the ferric state should raise the midpoint potential  $+193$  mV relative to Dap1p. The change in ferrous heme affinity also contributes to the reduction potential difference between these two proteins. The 5-fold, or 1 kcal/mol, destabilization of ferrous Dap1p<sup>Y138F</sup> relative to Dap1p should lower the midpoint reduction potential of Dap1p<sup>Y138F</sup> by 41 mV relative to Dap1p. Thus, the estimated reduction potential of Dap1p<sup>Y138F</sup> is  $-155$  mV, or  $-307$  mV  $+ 193$  mV  $- 41$  mV.

## Discussion

As discussed in the introduction, there is an ongoing debate in the literature regarding the biological role of yeast Dap1p. It has been proposed that Dap1p is involved in stabilizing protein levels of the P<sub>450</sub> enzyme, Erg11p, principally because deletion of Dap1p from the genome did not affect the RNA levels of Erg11p, but did affect both the Erg11p protein level and sterol synthesis (7). This hypothesis, however, was brought into question when Hughes et. al. observed that the protein levels of the human homologue of Erg11p (Cyp51A1) were unaffected by the absence of the human homologue of Dap1p (PGRMC1). Additionally, they reported a 1:1 stoichiometric protein-protein interaction between Dap1p and Erg11p, and found that *dap1Δ* cells transformed with the low affinity heme binding mutant, Dap1p<sup>Y138F</sup>, also showed lowered sterol synthesis, suggesting that heme binding was critical for the ability of Dap1p to rescue sterol synthesis (8).

In order to probe the role of heme binding and Dap1p function further, we embarked on a more detailed investigation of Dap1p. Initially, we probed the cellular properties of Erg11p and observed no change in mRNA, or protein levels in the absence of genomic DAP1, consistent with the data of Hughes et. al. for the human homologues (8). In addition, we observed that the  $t_{1/2}$  of Erg11p degradation was unchanged in *dap1Δ* strains. These results indicate that Dap1p does not stabilize Erg11p and weakens the case for Dap1p being a direct heme chaperone to Erg11p, as previously suggested (5–7).

Nevertheless, heme binding to Dap1p still appears to be required for ergosterol biosynthesis activity (8) and yet we previously reported that Dap1p, as isolated, only contained  $\sim 30\%$  bound heme (6). We resolved this inconsistency in the current investigation by achieving  $80 \pm 20\%$  heme loading for Dap1p with direct titration of free heme. This result indicated a tight affinity of the Dap1p for heme, however, the determination of the heme affinity to Dap1p ( $K_D$ ) was unsuccessful by direct titration due to its tight  $K_D$ . HSM apo-myoglobin was then titrated into Dap1p (30% heme loaded) as a competitive substrate, and the  $K_D$  was approximated to be  $\sim 0.1$  pM by singular value decomposition (14). This method assumes that the  $K_D$  of HSM apo-myoglobin, which has not been directly reported, is comparable to that of sperm whale myoglobin, whose  $K_D$  is  $\sim 0.01$  pM (16). Having narrowed down the  $K_D$  range, Dap1p was titrated with  $[\Delta 7\text{-His}]_2$ , a well characterized synthetic heme binding peptide ( $K_D$  of 160 pM),



and the  $K_D$  of Dap1p was determined to be  $400 \pm 200$  pM, with a 1:1 heme/protein ratio. This  $K_D$  value is significantly smaller than that expected for a non-specific hydrophobic pocket and supports the hypothesis that Dap1p is a heme binding protein (32). The ferrous heme affinity of Dap1p ( $K_D = 2 \pm 1$   $\mu$ M), however, is significantly weaker than that of ferric heme, which is consistent with expectations based on hard-soft acid-base theory for charged tyrosinate as the axial ligand (33). Our RR data on the ferrous-carbonyl complex supports this conclusion. Axial ligands compete for  $\sigma$ -bond donation to the iron, and the high  $\nu(\text{Fe-CO})$  frequency observed in Dap1p is indicative of a weak trans ligand.

The reduction potential of Dap1p was measured to be  $-307$  mV vs. SHE which is comparable to the  $-260$  mV value of bovine catalase, whose axial ligand is also a tyrosinate (29,30). This value is in the range of cytochrome  $P_{450}$  reductases,(34) making it tempting to suggest that Dap1p could provide electrons to Erg1p and hence increase the Erg1p activity in the cell with Dap1p present, as suggested by Hughes et al. (8). However, this hypothesis is unlikely since typical electron transport proteins, such as cytochrome b5, have similar coordination environments between their biologically relevant oxidation states (35). In addition, since the ferrous heme does not bind well to Dap1p, the heme would likely dissociate during redox catalysis (35). Finally, attempts to reduce the rat Dap1p homologue, IZA, by  $P_{450}$  reductases and cytochrome b5 have been unsuccessful *in vitro* (9).

Previously, we proposed that Y138 was the axial ligand based on the fact that the as-isolated phenylalanine mutant, Dap1p<sup>Y138F</sup> had no heme bound. We have subsequently been able to directly titrate Dap1p<sup>Y138F</sup> with free heme and have observed a 1:1 ratio of protein to heme, with a  $K_D$  of  $200 \pm 100$  nM. This affinity for heme is over 1000-fold weaker than that of Dap1p and is consistent with the loss of the native axial ligand, Y138 (32). The electronic spectrum of Dap1p<sup>Y138F</sup> with heme bound, however, is remarkably similar to that of Dap1p. This result possibly indicates either a loosely coordinated solvent molecule or an amino acid as a substitute axial ligand. The latter hypothesis is possible since there are a number of potential anionic ligands in close proximity within the hydrophobic pocket, as seen in the NMR structure of the non-heme bound, homologue At2g24940.1 (19). Interestingly, the  $K_D$  for Dap1p<sup>Y138F</sup> with ferrous heme is comparable to that of Dap1p ( $K_D = 10$   $\mu$ M), supporting the hypothesis that the ferrous heme iron is not coordinated to a strong axial ligand and that the hydrophobic pocket is the primary determinant for ferrous heme binding for both wild-type Dap1p and Dap1p<sup>Y138F</sup>.

The fact that Dap1p<sup>Y138F</sup> still binds ferric heme with a relatively strong affinity inspired us to investigate the functional activity of this mutant protein. Hughes et al. demonstrated that Dap1p<sup>Y138F</sup> lowered sterol synthesis in yeast similar to that of the *dap1Δ* strain, supporting the necessity of heme binding to Dap1p for Erg1p activation. They did not, however, demonstrate if Dap1p<sup>Y138F</sup> could rescue the growth sensitivity of *dap1Δ* in the presence of MMS or fluconazole. We therefore expressed Dap1p<sup>Y138F</sup> under the endogenous promoter, in a *dap1Δ* strain, and found that Dap1p<sup>Y138F</sup> did indeed rescue both phenotypes. This was a perplexing result because the data of Hughes et al. showed that ergosterol biosynthesis with the Dap1p<sup>Y138F</sup> mutant is much lower than wild-type (8). However, the data also indicated that the total amount of ergosterol was still greater in Dap1p<sup>Y138F</sup> than in *dap1Δ* and more importantly, the level of the toxic dienol (ergosta-5,7-dienol) was lower in the Dap1p<sup>Y138F</sup> strain than in the *dap1Δ* strain. Since it is unclear what ratio of ergosterol to dienol is required to manifest the MMS and fluconazole sensitivity, we hypothesize that the lowered heme binding of Dap1p<sup>Y138F</sup> is still sufficient for a low level of activity which rescues the growth phenotypes, but not enough to match the sterol synthesis of wild-type Dap1p, as measured by GC-MS (8). This result supports the hypothesis that the heme binding is required for function and the biological activity Dap1p is regulated by its ability to bind heme. It should also be noted that we performed the growth sensitivity experiment with Dap1p<sup>D91G</sup> and found no

rescue, similar to that seen by Mallory and coworkers (7). The result is most likely due to the fact that Dap1p<sup>D91G</sup> has an unstable protein structure, since we found no soluble Dap1p<sup>D91G</sup> when it is expressed in *E. coli*.

The importance of heme binding for Dap1p appears in conflict with published data suggesting that MAPR's bind sterols (1,3,19,36,37). Reports of progesterone binding are predominantly with the porcine homologue PGRMC1, which was measured to have two binding affinities to progesterone, 11 nM and 286 nM (1). There is additional evidence for regulation of rat PGRMC1 (also known as Vema, IZA, 25-Dx, and sometimes mPR) by progesterone and involvement of PGRMC1 in cellular, non-genomic progesterone responses (37–42). However, progesterone binding by rat PGRMC1, was later shown to be nonspecific and weak by Min et al (5). Instead, they found that rat PGRMC1 bound heme and activated the cytochrome P<sub>450</sub>, CYP21, a key enzyme of progesterone biosynthesis, similar to the biological function proposed by Hughes et al (8). These data indicate that MAPR's do bind heme and interact with P<sub>450</sub> enzymes, but it may also be possible that they have a dual function and bind sterols as well, possibly competitively with heme. Clearly more studies are needed to clarify the full biological role of MAPR proteins.

In summary, we have made several interesting contributions towards characterizing Dap1p's role as a heme binding protein. First, we have demonstrated that Dap1p does not directly regulate the Erg11p at any level, be it mRNA, protein, or protein degradation. Secondly, Dap1p preferentially binds ferric heme tightly at a 1:1 stoichiometry ( $K_D = 400$  pM) but ferrous heme poorly ( $K_D = 2$  μM), supporting its designation as a ferric hemoprotein. Thirdly, the RR results and the reduction potential of Dap1p are consistent with a 5-coordinate heme iron with tyrosinate axial ligation ( $E = -307$  mV). Finally, even though the weaker affinity of Dap1p<sup>Y138F</sup> for ferric heme ( $K_D = 200$  nM) lowers the production of ergosterol with respect to wild-type, it is still sufficient to rescue the growth sensitivity of *dap1Δ* to fluconazole and MMS, suggesting that the activity of Dap1p is directly related to its ability to bind heme and most likely not its redox potential.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Abbreviations

Dap1p, wild-type yeast Dap1 protein  
 Dap1p<sup>Y138F</sup>, Dap1p-Tyr138fPhe  
 Dap1p<sup>D91G</sup>, Dap1p-Asp91Gly  
 Erg11p, yeast Lanosterol 14-alpha-demethylase protein  
 Erg11p-HA, yeast Lanosterol 14-alpha-demethylase protein with a Hemagglutinin Tag on the C-Terminus  
 DAP1, wild-type yeast Dap1 gene  
 ERG11, yeast Lanosterol 14-alpha-demethylase gene  
 PGRMC1, progesterone receptor membrane component-1 protein  
 MAPR, membrane-associated progesterone binding protein  
 IZA, rat inner zone antigen  
 SD-His, synthetically defined media lacking histidine  
 PGK, phosphoglycerate kinase  
 AAA, amino acid analysis  
 ICP-MS, Inductively Coupled Plasma Mass Spectrometry  
 MMS, methyl methanesulfonate  
 Mb, myoglobin

apoMb, apomyoglobin  
HSM, horse skeletal muscle  
TBP, TATA box binding protein  
RR, Resonance Raman  
SHE, standard hydrogen electrode  
GC-MS, gas chromatography mass spectrometry

## Acknowledgement

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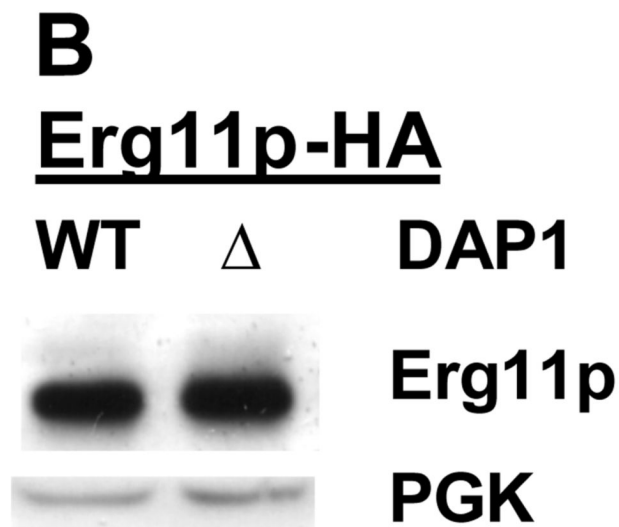
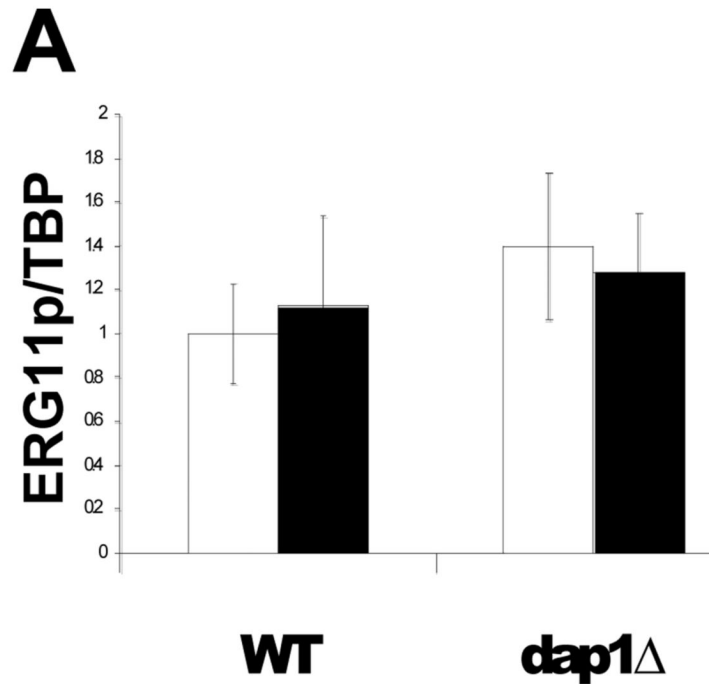
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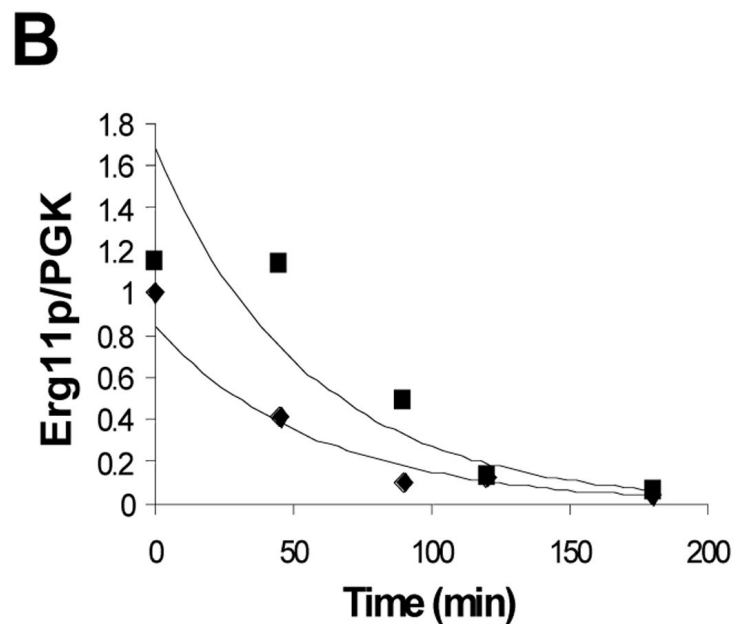
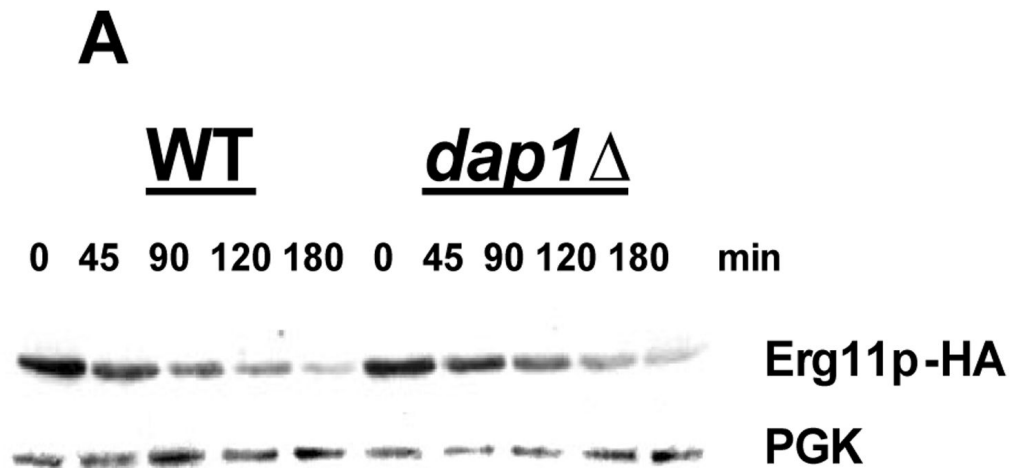
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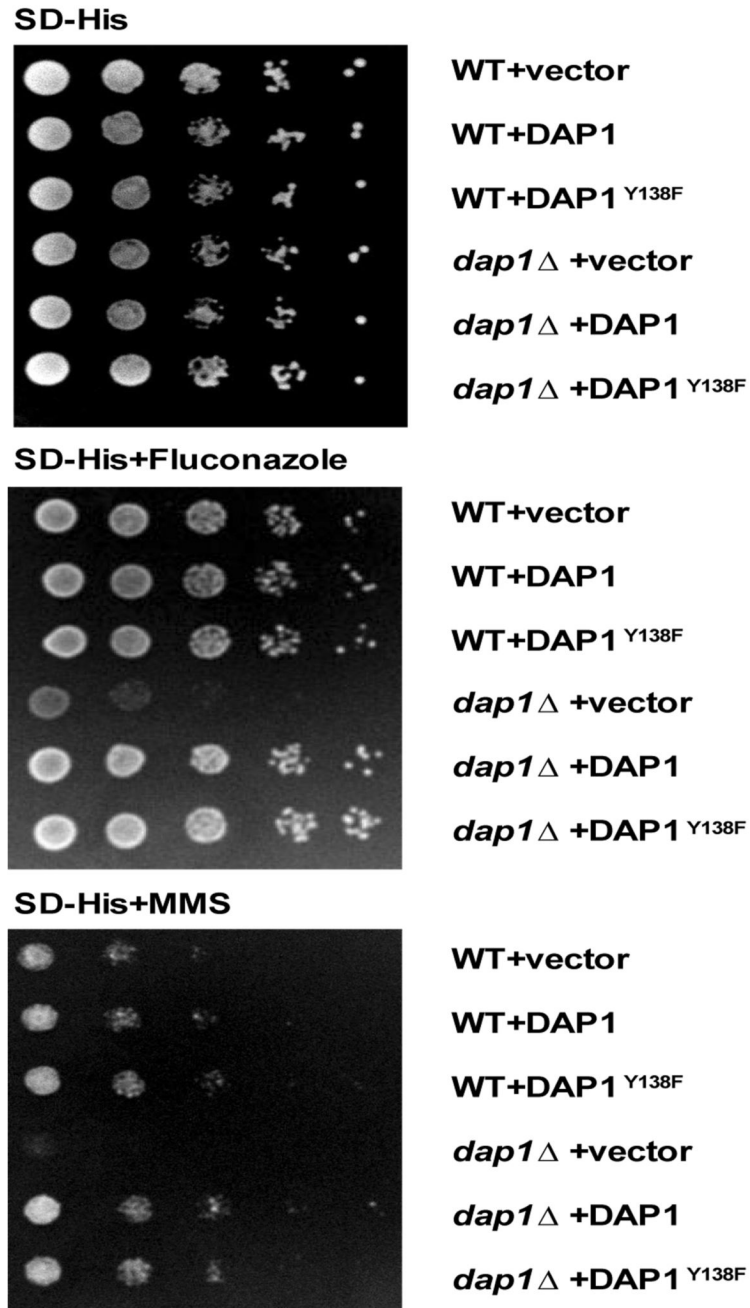


**Figure 1.**

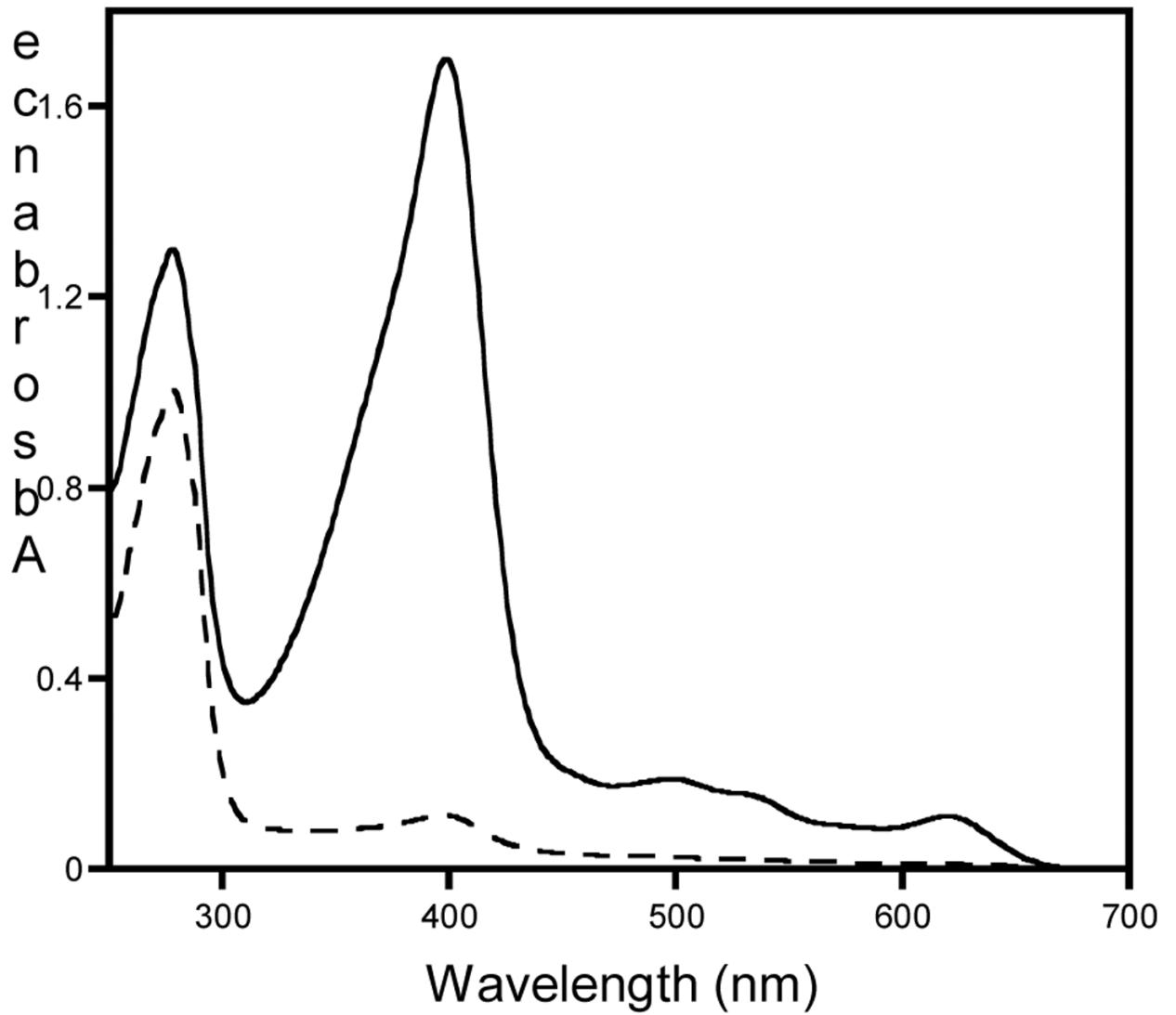
Wild-type and *dap1*Δ yeast strains were tested for A) DNA expression levels of ERG11, as measured by rtPCR (normalized to TBP), while grown with heme (black) and without heme (white) and B) Protein expression levels measured by western blot, without heme added.



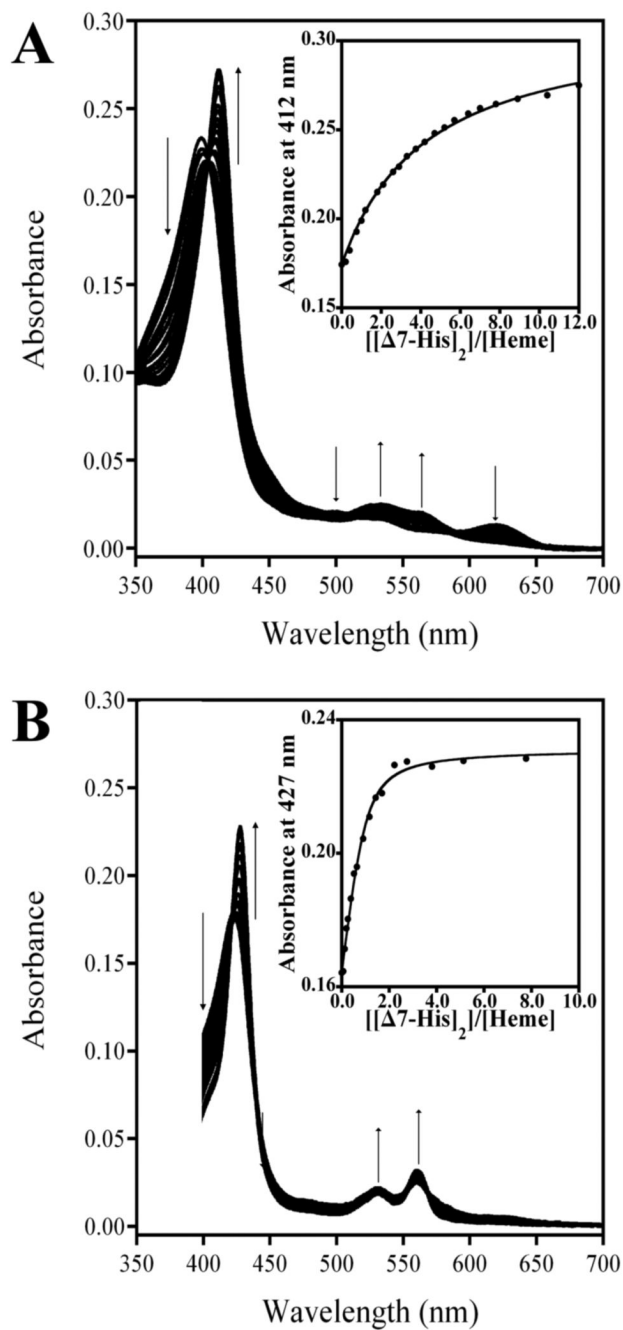
**Figure 2.** Measurement of  $t_{1/2}$  of Erg11 p in wild-type and *dap1*Δ yeast strains. A) Protein expression levels of Erg11p and PGK at 0, 45, 90, 120, and 180 mins after repression by glucose of Erg11 p-HA on the galactose promoter. B) Calculation of Erg11 p degradation rates, relative to PGK (♦)  $t_{1/2}$  = 40 mins and *dap1*Δ (▪)  $t_{1/2}$  = 38.



**Figure 3.** Growth of yeast strains wild-type (BY4741) or isogenic *dap1*Δ transformed with pRS313 vector containing either vector only, DAP1, or DAP1<sup>Y138F</sup>. Cells were serially diluted 1:10 and grown for 3 days at 30°C.

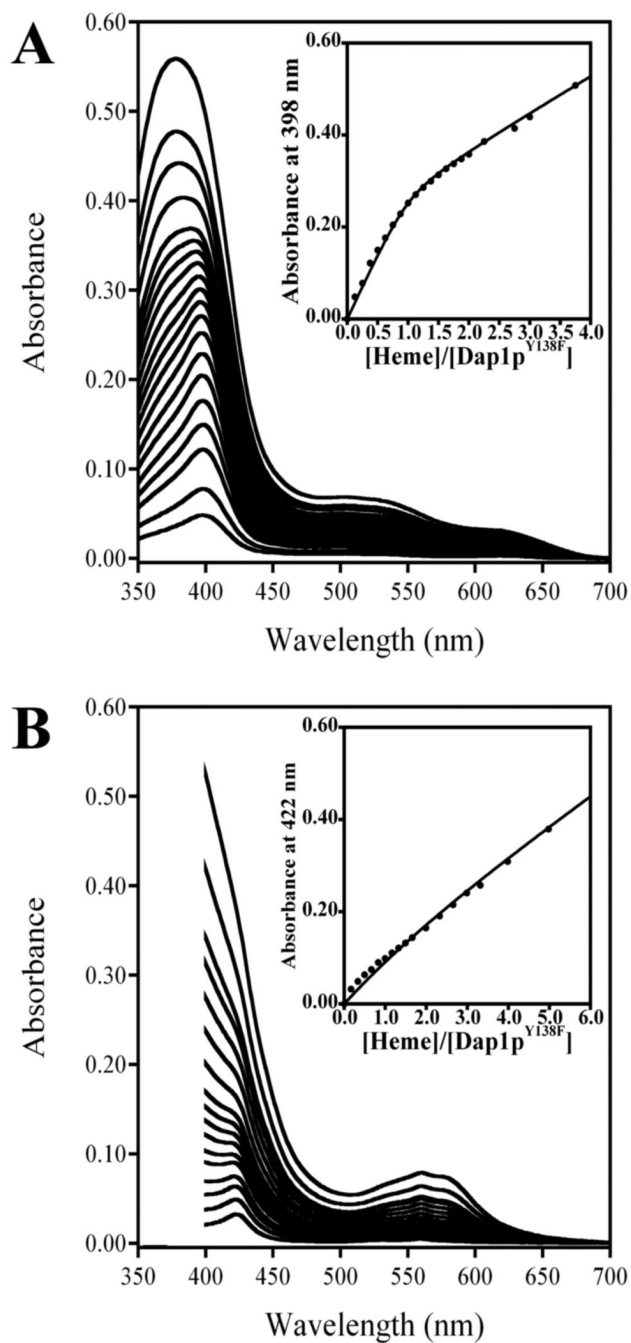


**Figure 4.** Representative UV-visible spectra of 1 mg/ml samples Dap1p (—) (as purified with ~20% heme bound) and Dap1p<sup>Y138F</sup> (---) (as purified with <2% heme bound).

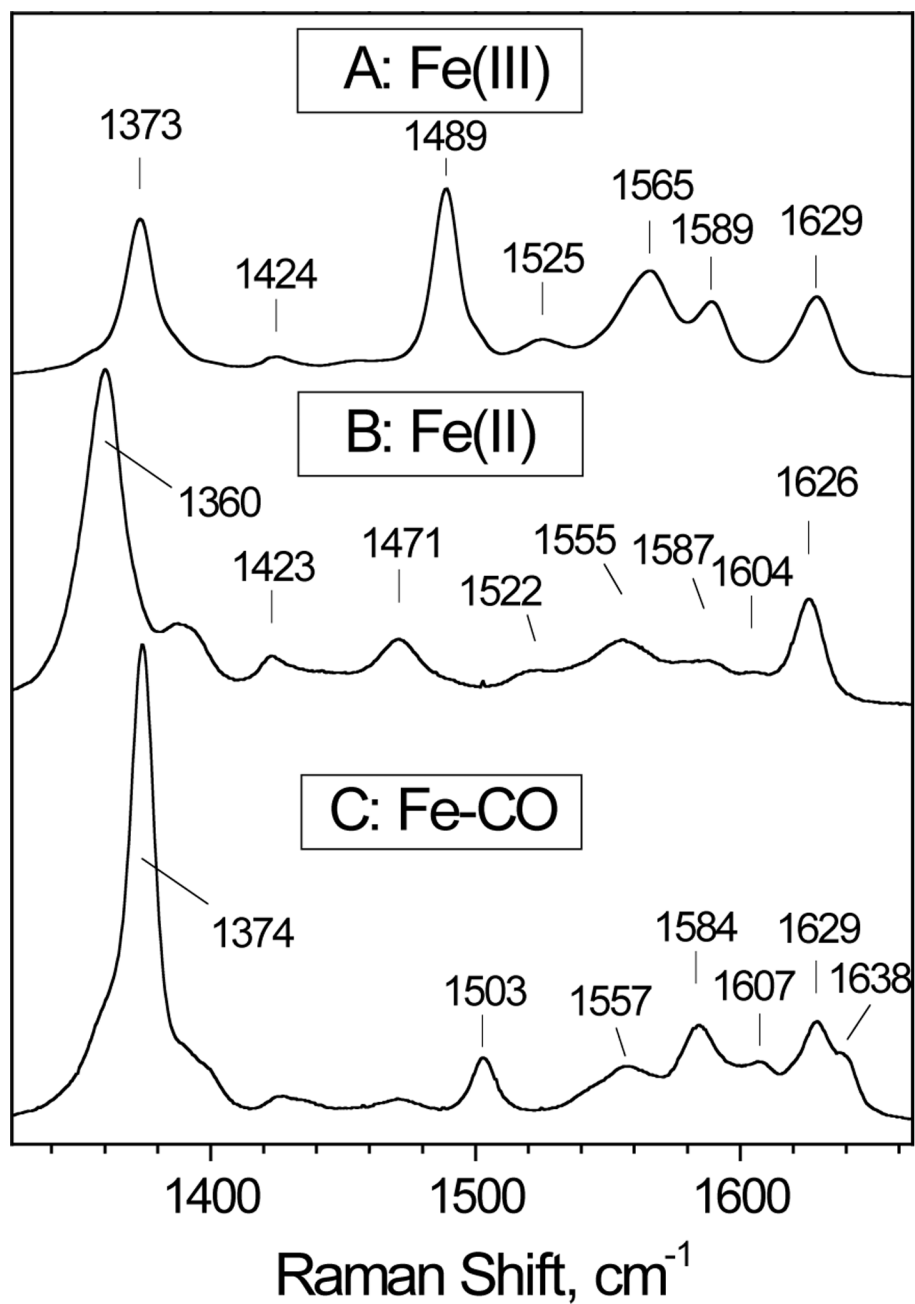


**Figure 5.** Representative competition titration of ferric (A) and ferrous (B) heme loaded Dap1p with  $[\Delta 7\text{-His}]_2$ . The insets, which show the increase in absorption at the Soret of heme loaded  $[\Delta\text{-His}]_2$ , indicate the transfer of heme from Dap1p to  $[\Delta 7\text{-His}]_2$ ; the change in absorption is fit to a competition model that gives the ferric and ferrous heme loaded Dap1 p  $K_D$  values.

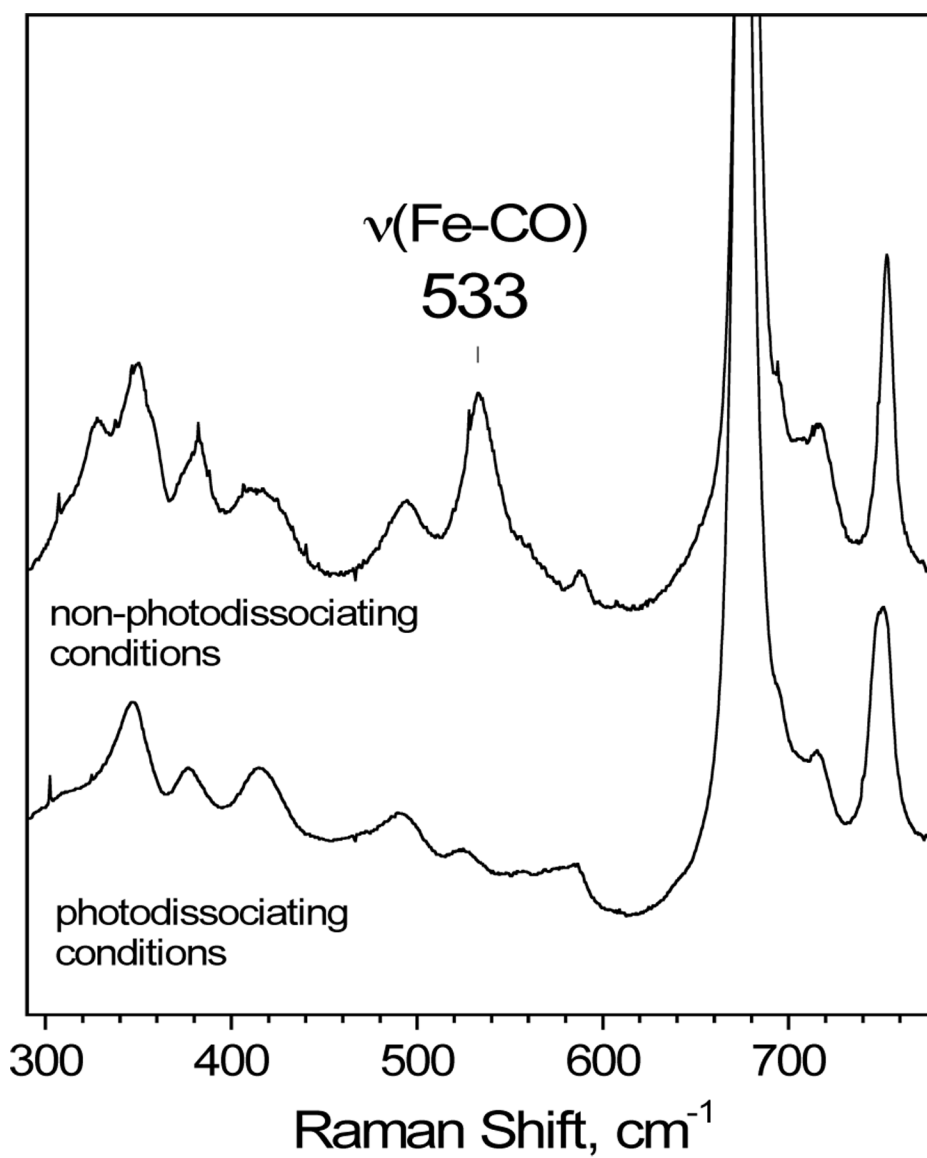




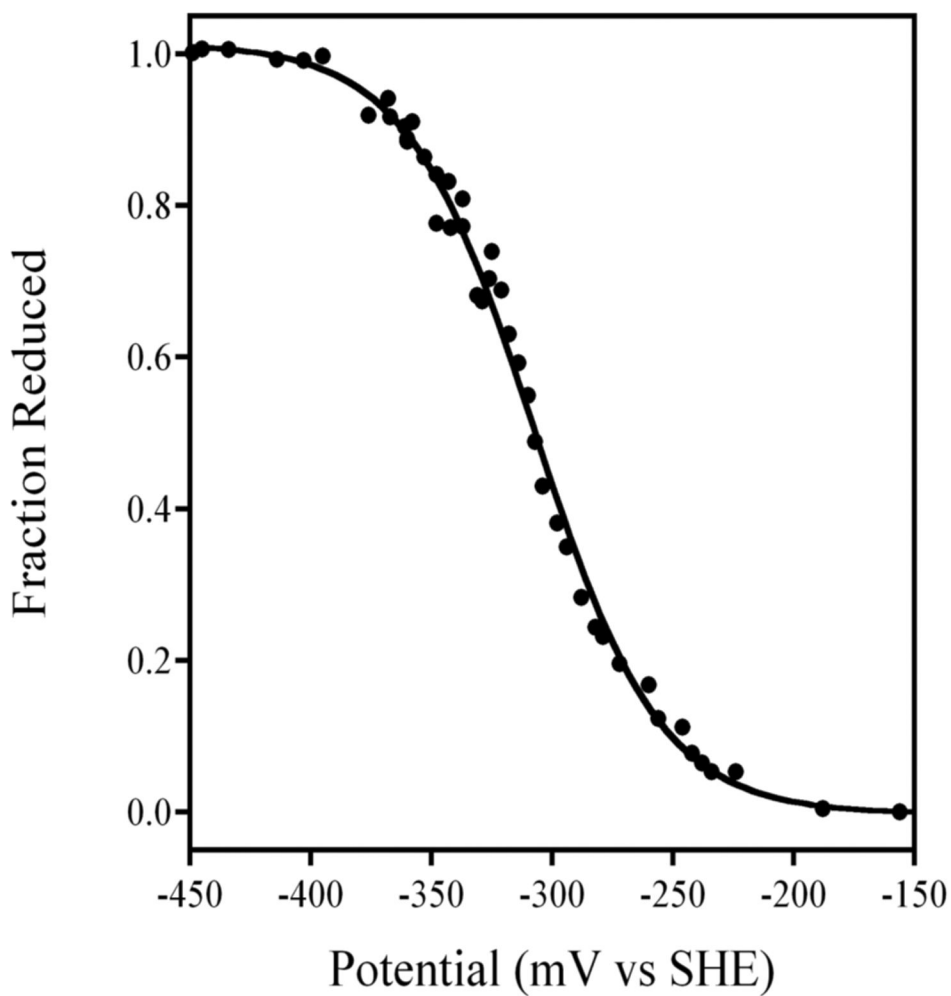
**Figure 6.** Direct titration of ferric (A) and ferrous (B) heme into Dap1p<sup>Y138F</sup>. Insets show the change in absorption at the Soret as a result of heme binding, and are fit to 1:1 binding models that give the ferric and ferrous  $K_D$  values of Dap1p<sup>Y138F</sup>.



**Figure 7.** High-frequency region of the RR spectra of Dap1p ferric (A), ferrous (B), and ferrous-CO complex (C) obtained at room temperature with a 413-nm excitation.



**Figure 8.** Low-frequency region of the RR spectra of the ferrous-CO complex of Dap1p obtained at room temperature with a 413-nm excitation. To achieve non-photodissociating conditions, the laser beam was defocused on the samples and kept below less than 1 mW using neutral density filter.



**Figure 9.** Redox potentiometry of heme loaded Dap1p. The change in absorbance at the Soret as a function of solution potential is best fit to an  $n = 1$  Nernst equation that gives a reduction potential of  $-307$  mV vs. SHE.