Purification of Dap1p, a Heme-Binding Damage-Associated Response Protein

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ABSTRACT

Saccharomyces cerevisiae damage-associated response protein (Dap1p) has been found to bind heme, similar to cytochrome b_5, through an axial ligand, Tyr138. The heme is noncovalently attached to Dap1p and it is thought to act as a chaperone, delivering it's heme to Erg11p/Cyp51p, a cytochrome P450 protein. Erg11p is the protein that the anti-fungal drug, Fluconazole targets in treating patients with antifungal infections. In this paper, a Hydrophobic Interaction Chromatography Column, a Size Exclusion Chromatography Column, UV-visable Spectra, and biochemical methods were used to purify wild type Dap1 and its mutant Y138F. The wild type Dap1 good heme content and its Y138F mutant did not have any heme bound.

Dap1p is a heme-binding protein of approximately 17kDa that contains a heme domain similar to that of cytochrome b_5 (1). Cytochrome b_5 is a heme-binding protein that regulates proteins that drive key steps in lipid metabolism (3). Dap1p (damage-associated response protein) is required for resistance to anti-fungal in Saccharomyces cerevisiae (1). An example of an anti-fungal drug is Fluconazole, which is used in treating patients with fungal infections. Fluconazole targets P450 protein Erg11p/Cyp51p in the sterol biosynthetic pathway in yeast (2). Sterols maintain an appropriate fluid membrane environment in response to changes in temperature (4). When Dap1p is removed from the cell or inhibited, it becomes more sensitive to fluconazole (2). Discovering an inhibitor drug for Dap1p could lead to better treatments and patients would be able to take lower levels of Fluconazole, and have fewer side effects.

Yeast cells with Dap1p present have been shown to contain high levels of Erg11p (2). Cells lacking Dap1p suffer from lower levels of Erg11p, which suggests that Dap1p may act as a chaperone delivering its heme to Erg11p whose activity as a P450 enzyme is known to be dependent on a prosthetic heme cofactor (3).

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In this paper we verify that Dap1p is a heme-binding protein and that its mutant Y138F eliminates heme-binding within the heme-1 domain. These findings support previous work demonstrating that Tyr 138 is the axial ligand bound to heme.

Methods: Protein Purification

Transformation. We transformed the E.coli BL21-(DE3) cells by inserting the pET-28a vector with "electroporation". Then we added 50µl of LB broth and incubated it for 30 minutes. Cells were transferred to a Kanamycin resistance plate where they grew overnight in the 37°C incubator.

Expression of Protein. The results of the above procedure were colonies of cells. One single colony of E.coli BL21-(DE3) was placed into 2mL LB broth tube and grew for 2-3 hours. Each tube was poured into a 2L flask containing LB broth and then Kanamycin was added (25µg/mL). The cultures were grown at 37°C (200rpm) until OD=0.6 was reached. Protein expression was induced by adding 0.5mM IPTG, and then cells were grown overnight at 20°C (100rpm) in the shaker.

Cell Lysis. The cells were spun down by centrifugation, and the pellet was resuspended in 50mL of 25mM HEPES cracking buffer (pH 7.5). The cells were lyzed by sonication.

Ammonium Sulfate Precipitation. Each solution was centrifuged at 4°C (18000 rpm) for 15 min. At 0% ammonium sulfate, we spun down the cells and discard the precipitate, which is the inner membrane. Then at 10% ammonium sulfate, the ammonium sulfate precipitates the smaller proteins ands separates them from bigger ones. We discarded the precipitate and kept the supernatant. At 40% ammonium sulfate, Dap1p is precipitates and separated from impurities. We stored the precipitate at -20°C and discard the supernatant.

Hydrophobic Interaction Chromatography Column. The protein was resuspended by adding minimal Milli-Q water. The Methyl hydrophobic interaction column was equilibrated at 40% ammonium sulfate 25mM HEPES buffer. Protein was then loaded into the column and fractions were collected. The good heme-to-protein ratio (A_{399}/A_{278}) fractions were collected and concentrated down in an Amicon.

Dyalysis: Protein solution was dialyzed overnight against 25mM HEPES pH 7.5 in order to get rid of small particles that are not wanted.

Size Exclusion Chromatography Column. The Column was equilibrated with 25mM HEPES pH 7.5, and then protein was loaded. 10mL of protein were loaded into the column and 3mL fractions were collected. Fractions were checked for purity by running a 15% SDS-PAGE with gels stained with Coomassie Blue. The darker and thicker fractions were collected, and concentrated down in the Amicon. After obtaining the purified protein we then added 5% glycerol to the pure protein and stored at -20°C. The mutant Y138F was also purified as described above.

UV-visable Spectra.

We measured the protein and heme concentrations by using Spectroscopy. The oxidized samples were reduced by adding Dithionite.

gel (Figure 2). A MW ladder was used to identify the protein.

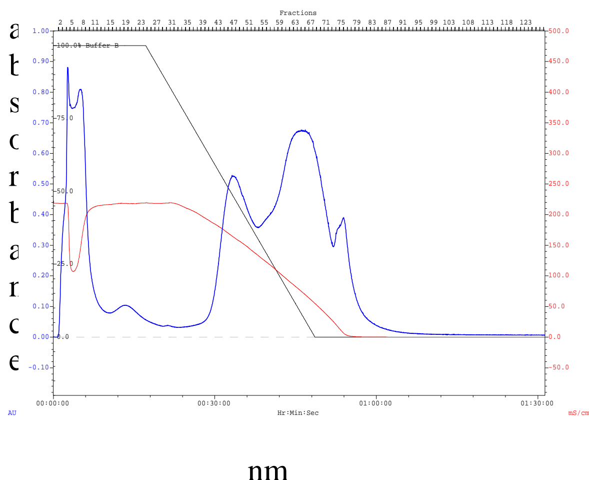

Figure 1: Hydrophobic interaction chromatography column shows the fractions of protein collected. Staring at 40% ammonium sulfate and eluted out to 0% ammonium sulfate.

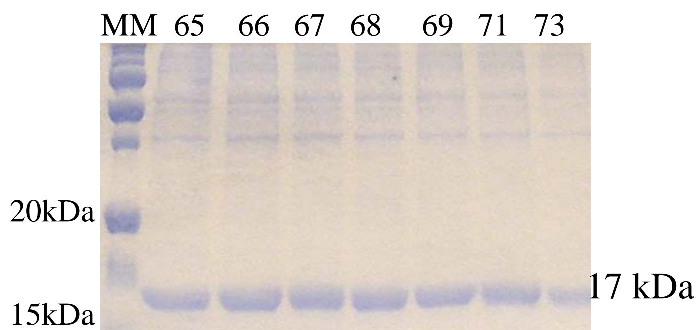

Figure 2: 15% SDS-PAGE GEL
The thick bands shown are Dap1, 17 kDa protein size. Used a Molecular Weight Marker for the ladder (MM). Fractions 67-71 were collected due to their higher heme-to-protein ratio (~0.5).

Results

Dap1p and Y138F were expressed in E.coli (BL21-DE3), and purified by ammonium sulfate precipitation, HIC column, and SEC column.

Hydrophobic Interaction Chromatography Column. The HIC column graph (Figure 1) shows the fractions at which the protein was eluted. Dap1 sticks at 40% ammonium sulfate and is eluted out at lower ammonium sulfate concentrations. Fractions were checked for purity by 15% SDS-PAGE

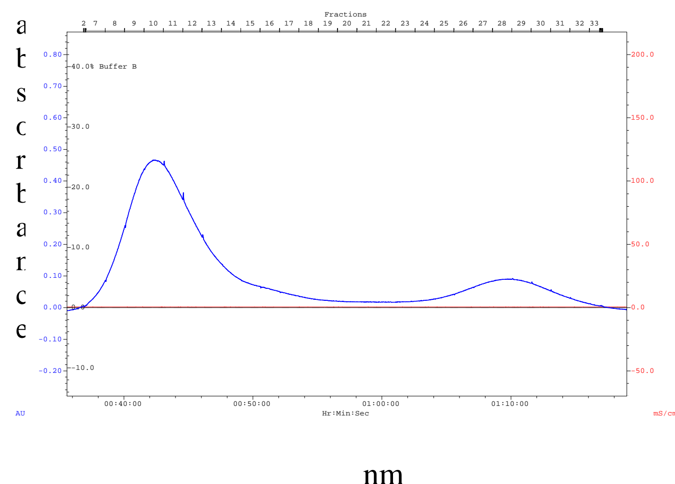

Figure 3: Size Exclusion Chromatography Column separated Dap1 from proteins smaller and/or greater than 17kDa and eluted out by fractions.

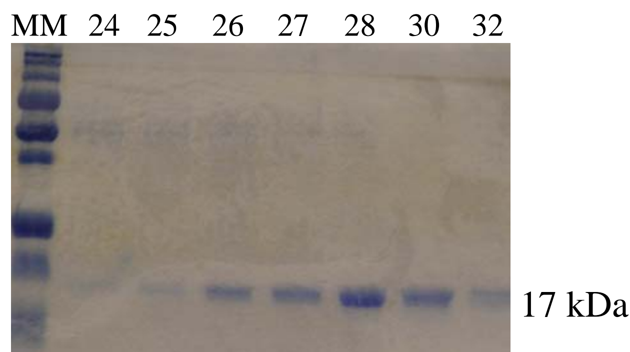

Figure 4: 15% SDS-PAGE GEL
The thick band shown in fraction 28 is the pure protein with the least amount of impurities. Fractions 26-30 were collected.

The good heme-to-protein fractions were diluted to 1:10 with Milli-Q water to check for the ratio by using UV-vis Spectra. We then took the ratio (A_{399}/A_{278}) of each fraction and pooled the ones with the good heme-to-protein ratio (~0.5). Fractions 67-71 were collected.

Size Exclusion Chromatography Column. Dap1p was separated from proteins greater or less than 17kDa. The

large peak in Figure 3 shows the larger proteins coming out first. The later peak corresponds to Dap1 pure protein that is eluted out around fractions 26-30, which we confirmed by 15% SDS-PAGE gel (figure 4). Fractions 26-30 were collected, and concentrated further with Amicon to about 7mL of pure Dap1p.

UV-Visable Spectra. The wild type Dap1p graph (figure 5) tells us that there is a heme-to- protein concentration in the pure sample. Protein absorbs light at 278nm and Heme absorbs light at 399nm. This graph confirms that Dap1p is a heme-binding protein. In contrast the mutant Y138F (figure 6) shows a larger concentration of protein than heme concentration, which confirms that this mutant is non-heme binding. The tiny curve at 400nm indicates that there is hardly any heme bound, since the side chain are charged the protein could be binding to a little heme.

DISCUSSION

Dap1p had been found to bind heme from previous biological studies, and in this paper we confirmed the heme-binding (1). The Y138F mutant was also confirmed to eliminate heme-binding. In this mutant the Tyrosine 138 was mutated to a Phenylalanine 138. Recent studies have suggested that Dap1p heme-binding is related to its function. It's believed that Dap1p acts as a chaperone delivering its heme to Erg11p in yeast. However the function of Dap1p has not been determined and/or proven yet. Researchers are working on discovering an inhibitor for Dap1p that could be used as a therapeutic target, in anti-fungal treatments and in chemotherapy treatment (2).

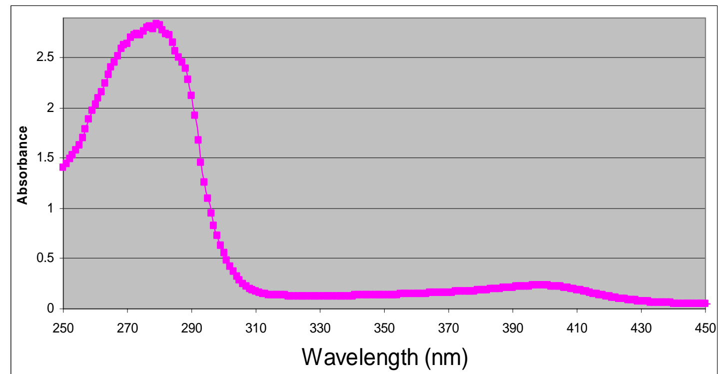

Figure 5. UV-visable Spectra of Dap1p. The peak to the left corresponds to protein absorbance at 278nm. The peak to t he right is the heme absorbance at 399nm.

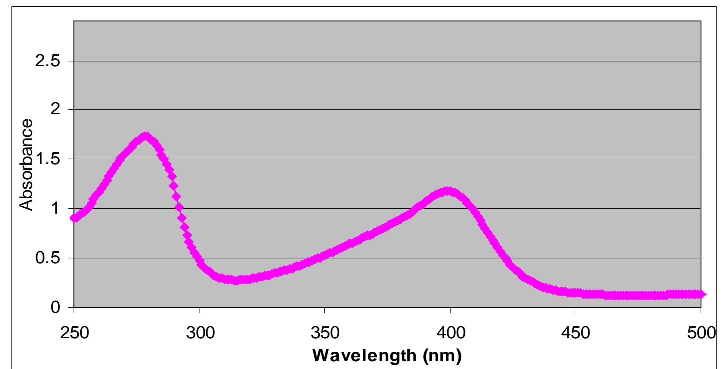

Figure 6. UV-visable Spectra of mutant Y138F. The peak to the left corresponds to the protein concentration in sample (278nm). The very tiny peak to the right corresponds to the Heme concentration in pure sample (399nm).

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