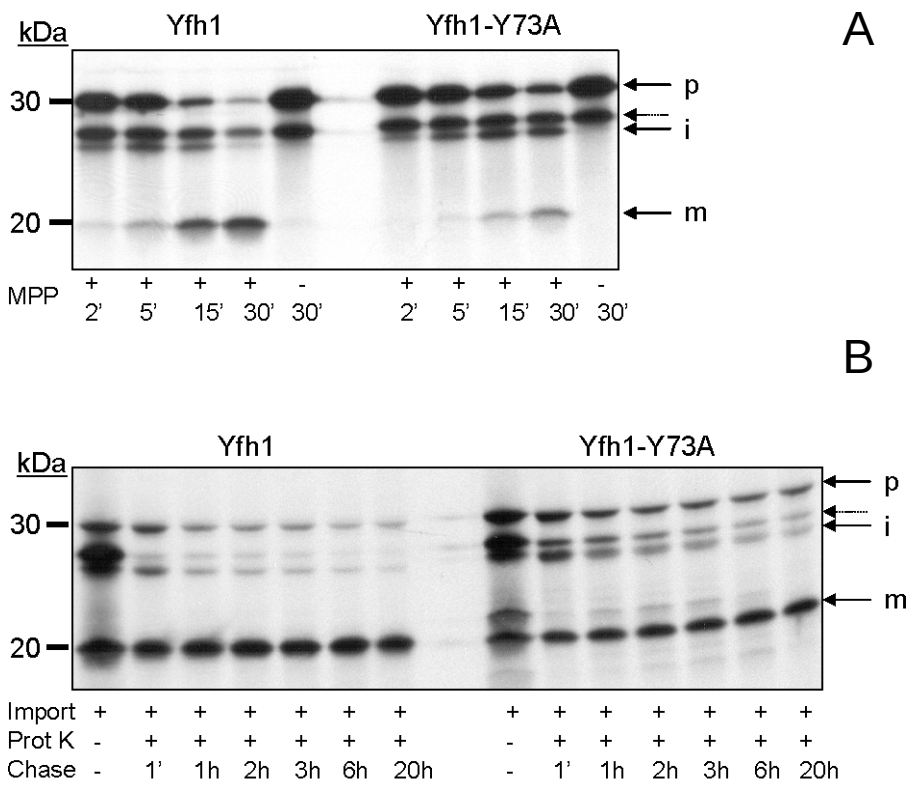
SUPPLEMENTAL DATA



Supplemental Fig. 1. **In vitro proteolytic processing and mitochondrial import assays of Yfh1-Y73A precursor**-The wild type *YFH1* and mutant *yfh1-Y73A* alleles were cloned in the *Eco*RI-*Sph*I sites of a pGEM-3Z vector under the T7 promoter control. *In vitro* transcription and translation were performed using the TNT Coupled Reticulocyte Lysate System (Promega) per manufacturer's protocol. Scintillation counting and SDS/PAGE were used to equalize radiolabeled Yfh1 and Yfh1-Y73A precursor proteins. Mitochondrial protein import and precursor processing assays were performed with freshly isolated yeast mitochondria and purified yeast mitochondrial processing peptidase (MPP) as described previously (1). **A** Translation was incubated with purified MPP at 30 °C for different times or without enzyme for 30 min. Aliquots were taken at the indicated time points and analyzed directly by 12% SDS-PAGE and autoradiography. p, i, m, Precursor, intermediate and mature forms of Yfh1p (2). Note the slower p→i→m kinetics of Yfh1-Y73A relative to wild type Yfh1. The dotted arrow indicates a product of internal translation initiation at Met 16 of the Yfh1 leader peptide, which generates a protein 5 residue longer than the normal processing intermediate. This product is processed in part by MPP, with slower kinetics in Yfh1-Y73A compared to Yfh1. **B** Translation was incubated with isolated yeast mitochondria at 30 °C (Import). Proteinase K (Prot K) treatment was performed to eliminate non-imported proteins, after which mitochondria were re-isolated in the presence of proteinase K inhibitor, and incubated at 30 °C (Chase). Aliquots were taken at the indicated time points and analyzed directly by 12% SDS-PAGE and autoradiography. Unprocessed p and i accumulate in Yfh1-Y73A mitochondria consistent with the processing defect observed in A. Some p→i→m conversion occurs during the first hour of the chase, after which the levels of mature form produced from the wild type or the mutant precursor remain essentially unchanged for up to 20 h.

Supplemental Table 1 – Biochemical properties of purified Yfh1-Y73A

	Buffer	Yfh1	Yfh1-Y73A
Iron Uptake[1] • Protein-bound iron at 60 min	2.2 ± 1.6[2] (8)	120 ± 5.0 (8)	97 ± 10.0 (3)
Ferroxidase Activity[3] • Fe^{2+}/O_2	3.8 ± 0.5[4] (3)	2.3 ± 0.3 (3)	2.9 ± 0.3 (6)
Fe^{2+} Delivery[5] • Heme	1.11 ± 0.47[6] (3)	11.03 ± 1.10 (3)	10.75 ± 1.27 (3)
• $Fe[BIPY]_3^{2+}$	5.38 ± 0.54[7] (3)	16.67 ± 0.82 (3)	16.97 ± 0.84 (3)

[1]Upon 60 min of aerobic incubation of protein (2 μM) with Fe^{2+} (150 μM) in 10 mM HEPES-KOH, pH 7.3, at 30 °C, each sample was centrifuged in an ultrafiltration device (5 kDa nominal molecular weight cut-off), and iron concentration was determined in the retentate (Protein Bound) as described previously in detail (3). [2]μM iron.

[3]Oxygen consumption curves were recorded with a MI-730 micro-O_2 electrode (Microelectrodes, Inc.) upon addition of 48 μM Fe^{2+} to buffer containing 96 μM protein or buffer without protein as described previously in detail (4). Conditions were 10 mM HEPES-KOH, pH 7.0, at 25 °C.

[4]Shown is the Fe^{2+}/O_2 stoichiometry determined for each completed reaction. An Fe^{2+}/O_2 ratio of ~2 is consistent with the ability to catalyze pair wise oxidation of Fe^{2+}; a Fe^{2+}/O_2 ratio of ~4 is expected for spontaneous iron autoxidation (4-6).

[5]Protein (3 μM) was aerobically loaded with 30 μM Fe^{2+} in 10 mM HEPES-KOH, pH 7.3, at 30 °C for 20 min. Iron-loaded protein was then aerobically incubated with either Bipyridin (BIPY) (2 mM), a chelator that preferentially binds Fe(II) (7), for 5 min, or yeast ferrochelatase and protoporphyrin IX (2 μM and 120 μM, respectively) for 20 min. The solution conditions used (aerobic buffer, pH 7.3, at 30 °C) normally promote the spontaneous conversion of Fe^{2+} to insoluble ferric iron oxides. In each case, the concentration of the final product, $Fe[BIPY]_3^{2+}$ or heme, was determined spectrophotometrically as described previously in detail (8). [6]μM heme; [7]μM $Fe[BIPY]_3^{2+}$. In all cases, data show the mean ± standard deviation. Numbers of independent measurements are given in parenthesis.

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