A Conserved 20S Proteasome Assembly Factor Requires a C-terminal HbYX Motif for Proteasomal Precursor Binding

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Supplementary Figure 1
Sequence alignment of the two DUF75 family members in *Methanococcus maripaludis* S2 encoded by open reading frames 0914 (COG1938; putative PbaA) and 1611 (COG2047; putative PbaB).
Supplementary Figure 2

(a) Sequence alignment of Pba2/PAC2 orthologs from across eukarya. Fungal Pba2 appears to contain a conserved HbYX motif (orange bar). In higher eukaryotes, PAC2 terminates in a conserved dipeptide consisting of a hydrophobic amino acid followed by tyrosine or phenylalanine (HbY/F motif).

(b) Deletion of PBA1 and/or PBA2 results in no observable phenotypes in vegetative growth of yeast.
**Supplementary Figure 3** High-copy mutant Pba1 and Pba2 proteins still fail to complement a *rpn4Δ pba1Δ pba2Δ* triple mutant. (a) Dilution series of indicated yeast strains transformed with high copy plasmids bearing the indicated Pba proteins, or with empty vector (v). (b) Western blots of lysates prepared from select strains from (a), namely *rpn4Δ pba1Δ pba2Δ* yeast strains transformed with empty high copy vector (lane 1), high copy vector bearing wild type Pba proteins (lane 2), or high copy vector bearing double HbYX mutant Pba proteins (lane 3). As a control, *rpn4Δ pba1Δ pba2Δ* yeast strain transformed with low copy vector bearing double HbYX mutant Pba proteins was included (lane 4) to demonstrate higher protein expression levels were achieved using the high copy plasmids.
Supplementary Figure 4 PbaA-PbaB complex(es) is not stable enough to survive non-denaturing electrophoresis. His-tagged archaeal proteins were expressed alone (lanes 1,2), or coexpressed (lanes 3,4), in *E. coli* and the lysates applied to Ni-NTA resin. The Ni-NTA eluates were separated by both SDS-PAGE (bottom) and native PAGE (top), and the proteins were stained with Gelcode Blue. No new bands were observed in lanes 3 or 4, suggesting that any complex(es) formed between PbaA and PbaB do not survive native PAGE.
Supplementary Figure 5 Ni-NTA fractionation of *E. coli* lysates.

**a.** Lysates of *E. coli* coexpressing His-tagged proteasome α subunit and either PbaA or PbaB were fractionated by Ni-NTA. T, total lysate; S, soluble fraction; P, insoluble pellet; F, flow-through; E, elute.

**b.** Ni-NTA eluates of His-tagged proteasome α or β subunit from lysates of *E. coli* that did not express PbaA or PbaB. Both a and b show Coomassie Blue-stained SDS gels.
Supplementary Figure 6 Using the βΔpro variant results in a proteasomal population without residual propeptide-containing β subunits.

Purified archaeal 20S proteasomes were separated by 11% SDS-PAGE and stained with Coomassie Blue. Two “wild-type” variants are indicated. In one variant, the β subunit contains its propeptide (lane 1). In the other variant, the β subunit lacks the propeptide (lane 2). A mutant proteasome in which the propeptide cannot be cleaved β(T1A) is also shown (lane 3). Note that in lane 1, a small amount of unprocessed β subunit remains (pro-β) relative to the majority of the β subunit which is correctly processed to the mature form (m-β). Lane 1 was overloaded for emphasis. Asterisk denotes C-terminally cleaved α subunit. Positions of molecular size standards (in kDa) are indicated.

Operon expressed:

- Lane 1: α-His β
- Lane 2: α-His βΔpro
- Lane 3: α-His β(T1A)
Supplementary Figure 7 Abrogaton of gel-shifts in K68A α subunit mutants. Lysates of *E. coli* expressing his tagged PHP, or his tagged mutant PHP (α K68A), were mixed with buffer (-) or with lysates of *E. coli* expressing untagged PbaA or untagged PbaA+PbaB. Mixtures were fractionated by Ni-NTA as described in Materials and Methods, and the eluates were separated by native PAGE on 4-10% gels. Gel shifted band is indicated by arrow. Positions of molecular weight standards (in kDa) are indicated to the right.
Supplementary Figure 8. Yeast Pba1-Pba2 is not a 20S proteasome activator.
(a) Peptidase activities of the wild-type yeast 20S proteasome in the absence or presence of Pba1-Pba2, or SDS control; activities were determined as described in the Methods. Error bars denote standard errors. Activity in the absence of Pba1-Pba2 is set to unity. The purified proteasomes were activation-competent since low levels of SDS could stimulate activity. Pba1-Pba2 denotes Pba1-His-Pba2.
(b) Purified yeast 20S proteasomes were incubated with recombinant Pba1-Pba2. At the indicated times, aliquots were withdrawn and mixed with 5X Laemmli sample buffer prior to SDS-PAGE and Western blotting with anti-His antibodies. Both Pba2 and the β4 20S subunit were tagged with hexahistidine.
Supplementary Figure 9. Stability of archaeal Pba proteins in the presence of 20S proteasomes.

(a-c) Purified archaeal 20S was incubated with purified PbaA (c), PbaB (d), or purified PbaA-PbaB complex (e) at 37°C. At the indicated times, aliquots were withdrawn and mixed with 5X Laemmli sample buffer prior to electrophoresis on 11% SDS gels. Proteins visualized with Gelcode blue. We verified that the archaeal 20S was still active at 90 minutes by determining that the rate of hydrolysis of model peptides remained constant (not shown).
Supplementary Figure 10. Generation and characterization of partially processed S1 and S2 proteasome intermediates.

(a) Schematic diagram of bacterial expression constructs developed to enable the recombinant production of the indicated species.

(b) The final steps of proteasome assembly entail the maturation of the inactive PHP, bearing the full complement of 14 β-subunit propeptides, into the fully active holo-20S, containing only mature β subunits. Implicit in this transition from PHP to holo-20S is the existence of short-lived maturation states containing intermediate numbers of processed β subunits. S1 and S2 were designed to mimic sub-populations of these maturation states. S2 represents a more mature set of species than S1 owing to the presence of two maturation-competent β-subunits encoded by the S2 operon, versus one maturation-competent and one maturation-incompetent (T1A) β subunit encoded by the S1 operon.

(c) Western blot of purified recombinant S2, probed with α-His antibody, to demonstrate incomplete processing of β subunit propeptides (arrowheads). Migration of mature β subunit (m-β) and precursor β subunit (pro-β) are shown for comparison. The blot only follows the processing of the β-His subunit in the S2 operon. Assuming that the β-His and the β subunits of this operon are incorporated with equal efficiencies, the extent of processing depicted here should be a reasonable approximation of the extent of processing of β subunits within S2 as a whole; the salient point being that the ratio of processed to unprocessed β subunits in S2 should be (considerably) greater than one.

(d) Peptidase activities of archaeal S1 in the absence or presence of the indicated archaeal Pba proteins were determined as described in the methods. Error bars denote standard errors. Activity of the S1-only sample is set to unity. Asterisks denote statistically significant differences from S1-only sample (**, p = 0.003; *, p = 0.013; n = 3).
Supplementary Figure 11: A repeat of the gel shift assay for PbaA binding to the precursor populations S1 and S2. Clear binding is only detected with S1 (lane 4), which has a higher fraction of particles with unprocessed beta subunits. It is possible that there is very weak binding to S2 (lane 2), which would also be consistent with PbaA binding to proteasomal precursors (particles in the more fully matured S2 population will have fewer remaining propeptides than S1; see Supplemental Fig. 10 for explanation).
Supplementary Figure 12
Sequence alignment of archaeal and actinomycete DUF75 superfamily members, represented by PbaA from *Methanococcus maripaludis* and a protein of unknown function from *Corynebacterium glutamicum* respectively. The HbYX motif in the archaeal protein is underlined. Note the C-terminal extension of the actinomycete protein.
Table I: Strains used in this study

| Name         | Genotype                                                                 |
|--------------|---------------------------------------------------------------------------|
| MHY500\(^1\) | \(\text{MAT}^a\) his3-\(\Delta\)200 leu2-3,112 ura3-52 lys2-801 trp1-1 gal2 |
| MHY501\(^1\) | \(\text{MAT}^a\) his3-\(\Delta\)200 leu2-3,112 ura3-52 lys2-801 trp1-1 gal2 |
| MHY794\(^1\) | \(\text{MAT}^a\) doa3\(\Delta\):HIS3 [YCplac22 doa5-1]                    |
| MHY1659\(^2\) | \(\text{MAT}^a\) his3-\(\Delta\)200 leu2-3,112 lys2-801 trp1\(\Delta\)63 ura3-52 PRE1\(^{FH}\):Y elbow211 (URA3) |
| MHY3410\(^3\) | \(\text{MAT}^a\) doa3\(\Delta\):HIS3 [YCplac22-DOA3-His6]                  |
| MHY4299\(^4\) | \(\text{MAT}^a\) pba4\(\Delta\):hphMX                                      |
| MHY4326       | \(\text{MAT}^a\) pba1\(\Delta\):kanMX                                     |
| MHY4328       | \(\text{MAT}^a\) pba2\(\Delta\):natMX                                     |
| MHY4330\(^4\) | \(\text{MAT}^a\) pba1\(\Delta\):kanMX pba2\(\Delta\):natMX                 |
| MHY5113       | \(\text{MAT}^a\) doa5\(\Delta\):HIS3 pba1\(\Delta\):kanMX [YCplac22 doa5-1] |
| MHY5115       | \(\text{MAT}^a\) doa5\(\Delta\):HIS3 pba2\(\Delta\):natMX [YCplac22 doa5-1] |
| MHY5117       | \(\text{MAT}^a\) doa5\(\Delta\):HIS3 pba1\(\Delta\):kanMX pba2\(\Delta\):natMX [YCplac22 doa5-1] |
| MHY5197       | \(\text{MAT}^a\) rpn4\(\Delta\):hphMX                                    |
| MHY5310       | \(\text{MAT}^a\) rpn4\(\Delta\):hphMX pba1\(\Delta\):kanMX                |
| MHY5311       | \(\text{MAT}^a\) rpn4\(\Delta\):hphMX pba2\(\Delta\):natMX                |
| MHY5312       | \(\text{MAT}^a\) rpn4\(\Delta\):hphMX pba1\(\Delta\):kanMX pba2\(\Delta\):natMX |
| XL210\(^3\)   | \(\text{MAT}^a\) doa3\(\Delta\):HIS3 ump1::pRS305-UMP1-HA2 pba1\(\Delta\):kanMX, pba2\(\Delta\):natMX [pRS317-DOA3-His6] |

MHY500 and MHY501 are wild-type strains and the remaining strains are congenic derivatives.
All strains are from this study, except:
1. Chen and Hochstrasser (1995)
2. Verma et al. (2000)
3. Li et al. (2007)
4. Kusmierczyk et al. (2008)
Table II: Plasmids used in this study

| Name  | Genotype                                      |
|-------|-----------------------------------------------|
| AKB191| pET42 psmA-his                                |
| AKB424| pET42 his-\(PBA2\) \(PBA1\)                   |
| AKB427| pET42 pbaA his-pbaB                           |
| AKB429| pET42 pbaA pbaB                               |
| AKB430| pET42 psmA psmB-his                           |
| AKB434| pET42 pbaA                                    |
| AKB435| pET42 his-pbaA                                |
| AKB436| pET42 pbaB                                    |
| AKB439| pET42 his-pbaA pbaB                           |
| AKB441| pET42 pbaA psmA-his                           |
| AKB442| pET42 pbaB psmA-his                           |
| AKB458| pET42 his-pbaADHbYX pbaB                      |
| AKB460| pET42 his-pbaB                                |
| AKB463| pET42 psmA psmB                               |
| AKB464| pET42 psmA-his psmB                           |
| AKB490| pRS316 his-\(PBA2\)                           |
| AKB493| pRS326 his-pba2(L265A)                        |
| AKB494| pRS316 his-pba2(Y266A)                        |
| AKB495| pRS316 flag-\(PBA1\)                         |
| AKB512| pRS316 flag-pba1(Y275A)                       |
| AKB513| pRS316 flag-pba1(L274E)                       |
| AKB528| pET42 psmA psmB(T1A)-his                      |
| AKB531| pRS316 flag-\(PBA2\) his-PBA2                 |
| AKB532| pRS316 flag-pba1(Y275A) his-PBA2              |
| AKB533| pRS316 flag-pba2(Y266A)                       |
| AKB534| pRS316 flag-pba1(Y275A) his-pba2(Y266A)       |
| AKB570| pET42 psmA(K68A) psmB(T1A)-his                |
| AKB572| pET42 psmA-his psmB(T1A)                      |
| AKB583| pET42 psmA psmB-his psmB                      |
| AKB584| pET42 psmA psmB(T1A)-his psmB                 |
| AKB594| pET42 his-pbaAΔHbYX                          |
| AKB595| pET42 his-pbaBΔC                              |
| AKB628| pET42 psmA-his psmBΔpro                       |
| AKB629| pET42 psmA psmBΔpro-his                       |
| AKB632| pET42 psmA(K68A)-his psmB(T1A)                |
| AKB636| pET42 pbaAΔHbYX pbaB                         |
| AKB637| pET42 pbaAΔHbYX                              |
| AKB638| pET42 pbaA pbaBΔC                            |
| AKB639| pET42 pbaBΔC                                  |
| AKB650| pET42 psmAΔN-his psmB(T1A)                    |
| AKB651| pRS426 flag-\(PBA1\) his-PBA2                 |
| AKB652| pRS426 flag-pba1(Y275A) his-pba2(Y266A)       |

Notes:  
psmA = \textit{M. maripaludis} \(\alpha\) subunit  
psmB = \textit{M. maripaludis} \(\beta\) subunit  
all plasmids generated in this study
SUPPLEMENTARY METHODS

Analysis of Pba1-Pba2/PAC1-PAC2 primary sequence

Initially, we surveyed the Conserved Domain Database (CDD)\(^1\) and found Pba2 and PAC2 annotated to the Domain of Unknown Function (DUF75) superfamily. A BLAST search versus archaeal sequences using PAC2 as input returned a number of uncharacterized archaeal proteins, with the top hits belonging to members of this superfamily (not shown). In addition, iterative BLAST (PSI-BLAST) with PAC1 also returned uncharacterized archaeal proteins in the DUF75 superfamily. In general, we found that all archaea encode at least 2 DUF75 superfamily members. According to the Clusters of Orthologous Groups of Proteins (COG) database\(^2\), archaeal DUF75 superfamily members cluster into two orthologous groups: COG1938 and COG2047. The former are found in all archaeal genomes examined, and the latter are found only in the Euryarchaeota. However, those archaea that do not contain a COG2047 superfamily member, encode two COG1938 superfamily members, such that the number of distinct DUF75 species within each archaeal genome is always at least 2 (there are some species that contain more than two, for example two COG1938 and one COG2047, but the significance of this is not clear). When present within the same species, COG1938 and COG2047 share ~23% sequence identity (Supplementary Fig. 1).

Generation of wild-type variant (βΔpro) used in all experiments

Unless otherwise stated, in all our experiments wild-type (WT) archaeal proteasome was recombinantly generated in *E. coli* from an operon consisting of an α subunit and a β subunit variant (βΔpro). The βΔpro protein was previously shown to be fully competent for assembly\(^3\). It was used here to ensure that all β subunits were in their mature processed form as expression of the full-length β subunit in *E. coli* invariably resulted in a small fraction of β subunits remaining unprocessed (Supplementary Fig. 4). The βΔpro WT proteasome assembled into a species that migrated similarly to the 670 kDa standard and exhibited robust peptidase activity against a fluorogenic peptide substrate (Fig. 4b in main text of article).

Role of Pba1 and Pba2 HbYX motifs in yeast

The immunoblot analysis in Fig. 2c (main text) was repeated several times, and whereas the levels of the Pba2 protein always remained unchanged regardless of whether Pba2 was wild-type or mutant, the levels of the Pba1 protein in the double HbYX mutant samples were slightly variable between repetitions of the experiment (not shown). To rule out reduced Pba1 protein level as a factor in the synthetic growth defect, the different pba alleles were subcloned into high-copy plasmids and the complementation analysis with the pba1Δpba2Δrpn4Δ triple mutant was repeated. The high-copy mutant pba1-pba2 plasmid also failed to complement the mutant strain even though Pba1 was expressed at a much higher level than was observed with the low-copy plasmid (Supplementary Fig. 3b).

To provide biochemical evidence for the importance of the Pba1-Pba2 HbYX motifs in proteasome precursor binding in yeast, we carried out co-precipitation analysis in strains expressing wild-type Pba1-Pba2 or Pba1-Pba2 with a point mutation in each HbYX motif (Fig.
3b). The yeast XL210 strain was transformed with pRS316, AKB531, or AKB534 and grown in selective media. The protocol for proteasome immunoprecipitation was adapted from Verma et al., 2000. A 1-L culture at OD₆₀₀ 1.0 was pelleted and ground under liquid nitrogen. The resulting powder was stored at -80°C. The powder was thawed in Buffer AY supplemented with 2 mM ATP, and the lysate was centrifuged for 20 min at 34,310 x g at 4°C. The supernatant was filtered through cheesecloth to remove lipids. Protein concentration was determined with the BCA assay, and 5 mg of total protein was applied to 100 µL of Flag M2 agarose beads for 1 hr on a rotating wheel at 4°C. The beads were then washed once with 1 mL Buffer AY containing 2 mM ATP and 0.2% Triton X-100. The beads were further washed twice with 1 mL Buffer AY with 2 mM ATP. Bound proteins were eluted for 30 min at room temperature with end-over-end rotation in 200 µL Elution Buffer (25 mM Tris-HCl, pH 7.5; 150 mM NaCl; 15% Glycerol; 5 mM MgCl₂; 2 mM ATP; 100 µg/mL 3XFLAG peptide). 50 µg of total protein was loaded for both SDS PAGE and nondenaturing PAGE analysis (Kusmierczyk et al., 2008.) Standard protocols were used for subsequent immunoblot analysis.

**Generation of S1 and S2 assembly intermediates**

Two operons were generated to produce two distinct archaeal proteasome intermediate populations (Supplementary Fig. 10a,b). The first operon contained ORFs for archaeal α subunit, a β-His6 subunit, and an untagged β subunit. Expression of this operon gave rise to a set of proteasomal species (S2) with a small but appreciable fraction of unprocessed β subunits (Supplementary Fig. 10c, last lane). The average ratio of processed to unprocessed β subunits in these species (S2) should be greater than one. The second operon contained ORFs for archaeal α subunit, mutant β(T1A)-His6 subunit, and untagged β subunit. Assuming that β(T1A) and β subunits incorporate with equal efficiencies, the result would be a binomially distributed collection of 20S particles with an average of 7 immature β(T1A) and 7 maturation-competent β subunits per particle. However, since wild-type β subunits do not undergo complete processing in *E. coli* (Supplementary Figs. 6 and 10c), the average ratio of processed to unprocessed β subunits in these proteasome species (S1) should be (somewhat) less than one. Thus, this second operon should produce, on average, a less fully matured or “earlier” set of proteasomal maturation intermediates (S1) relative to the first operon (S2).
The HbYX motif of Pba2 is only conserved among fungal orthologs, but several findings suggest that the C-terminus of Pba2/PAC2 in other eukaryotes is similarly important. First, despite lacking a HbYX motif, all non-fungal orthologs of Pba2/PAC2 contain a highly conserved C-terminal dipeptide, Hb-Y/F. Second, the C-termini of all three PA28/11S activator subunits terminate with a similar conserved HbY dipeptide, which mediates their interactions with the 20S proteasome α-ring pockets. Finally, the PbaB C-terminus is required for PbaB binding to inhibitor-treated proteasomes even though it lacks a canonical HbYX motif.

We also suspect that Pba1 and Pba2 adopt a similar overall fold despite lacking obvious sequence similarity. The reasoning is as follows. All archaea contain at least two DUF75 superfamily members, and we have shown that for *M. maripaludis*, a species that encodes both a COG1938 (PbaA) and COG2047 (PbaB) member, the two proteins form a complex. The eukaryotic orthologs are also made of protein products from two genes, and the yeast proteins contain functionally redundant HbYX motifs. As noted above, mammalian PAC2 might have a functional near-HbYX motif, thereby maintaining this functional duality. Structural homology would thus be the most parsimonious explanation for two nearly identically sized proteins with functionally redundant C-terminal HbYX motifs. In support of this idea, iterative BLAST searches against archaeal genomes using human PAC1 as input return weakly scoring sequences that are DUF75 superfamily members.

The S1 and S2 archaeal 20S precursors were generated to control for the negative result obtained with the peptidase activity assay using mature 20S (Fig. 5c). The latter result suggested that archaeal Pba proteins do not function as activators of mature 20S. However, in the absence of a positive control with a known activator, the lack of activation by Pba proteins might simply mean the assay was not reliable. Using PAN as a positive control was not possible, owing to the known inability of PAN to stimulate the hydrolysis of fluorogenic tetrapeptide substrates; our attempts to use the longer LFP substrate were unsuccessful, and low levels of SDS were similarly ineffective (data not shown). PHP binds PbaA well but does not have peptidase activity owing to the β-T1A mutation. Mature 20S has robust peptidase activity, but appears not to bind PbaA. Therefore, we reasoned that if we generated a proteasome species that was intermediate in processing state between PHP and mature 20S, we might get a species that retains some binding affinity for Pba (owing to the presence of some propeptides) while at the same time containing some mature β subunits whose peptidase activity we could measure. Weak S1 and S2 activation by PbaA allowed us to conclude that the peptidase assay could reliably report on potential PbaA activator activity. Therefore, the fact that mature 20S was not activated by the Pba proteins was not due to a deficiency in the assay system.

The need to protect α ring-containing intermediates from premature association with activators may reflect evolutionarily conserved features of 20S assembly shared by archaea and eukaryotes. Interestingly, DUF75 proteins are also found in actinomycete bacteria, organisms that also encode 20S proteasomes. However, bacterial DUF75 proteins are C-terminally extended (Supplementary Fig. 12) and do not terminate with HbYX motifs, implying different mechanism(s) of action. Actinomycete proteasomes assemble by a pathway(s) that is different from their archaeal/eukaryotic counterparts: their α subunits are believed to heterodimerize with β subunits first, and these α/β units then assemble into half-proteasomes. Since actinomycete proteasome assembly bypasses most full α ring-containing intermediates, there might have been
less selective pressure for a mechanism to limit activator-20S precursor binding. Nevertheless, actinomycete DUF75 proteins may still have a function in proteasome assembly and/or proteasome function.

Two crystal structures of bacterial (actinomycete) DUF75 proteins are available (PBD: 2P90; and ref. 10). Recently, a crystal structure of the archaeal PbaA homolog from *Thermoplasma acidophilum* (PDB: 3GAA) was deposited, although no publication accompanied the structure. The archaeal and bacterial subunits in the three structures share a similar overall fold, consistent with their membership in the same superfamily. The bacterial proteins crystallized as trimers while the archaeal protein seemed to form a dodecamer. The physiological relevance of these quaternary structures is not known. The archaeal dodecamer is a dimer of ring-shaped pentamers with the C-termini exposed to solution. Formation of a ring-shaped complex by PbaA opens up the possibility that this protein functions as a platform or scaffold in proteasome assembly. However, it also raises the question of why the eukaryotic orthologs exist as dimers. The answer might be related to the subunit heterogeneity of eukaryotic α rings. For binding of the homomeric archaeal α ring to a PbaA ring, or even to a PbaA-PbaB ring (if such a structure exists), binding register is irrelevant since all α subunits are equivalent. However, with the heteroheptameric eukaryotic α ring, there could be pressure to simplify a heteromeric assembly factor (for instance to a heterodimer) in order to maintain a specific binding orientation. Though the nature of the functional archaean species remains to be determined, the *Thermoplasma* PbaA crystal structure does show solvent accessibility of all C-termini, suggesting that the HbYX motifs are available for interaction with 20S, as suggested by our data. Using our *in vitro* system, the crystal structures can serve as useful starting points for mutagenesis studies to address the nature of the proteasome-bound Pba species.
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