# Six-fold rotational symmetry of ClpQ, the *E. coli* homolog of the 20S proteasome, and its ATP-dependent activator, ClpY

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**Abstract ClpQ (HslV) is a homolog of the β-subunits of the 20S proteasome. In *E. coli*, it is expressed from an operon that also encodes ClpY (HslU), an ATPase homologous to the protease chaperone, ClpX. ClpQ (subunit $M_r$ 19 000) and ClpY (subunit $M_r$ 49 000) were purified separately as oligomeric proteins with molecular weights of ~220 000 and ~350 000, respectively, estimated by gel filtration. Mixtures of ClpY and ClpQ displayed ATP-dependent proteolytic activity against casein, and a complex of the two proteins was isolated by gel filtration in the presence of ATP. Image processing of negatively stained electron micrographs revealed strong six-fold rotational symmetry for both ClpY and ClpQ, suggesting that the subunits of both proteins are arranged in hexagonal rings. The molecular weight of ClpQ combined with its symmetry is consistent with a double hexameric ring, whereas the data on ClpY suggest only one such ring. The symmetry mismatch previously observed between hexameric ClpA and heptameric ClpP in the related ClpAP protease is apparently not reproduced in the symmetry-matched ClpYQ system.**

*Key words:* Clp protease; HslU/HslV; ATP-dependent protease; Proteasome; Rotational symmetry; Image analysis

## 1. Introduction

Substrate selection by the Clp family of ATP-dependent proteases is determined by a set of specific ATP-dependent molecular chaperones that interact with and activate proteolytic components [1–3]. In *E. coli*, two ATP-dependent molecular chaperones, ClpA and ClpX, activate ClpP, an oligomeric protein that by itself displays only limited peptidase activity against low molecular weight peptides [4]. With ClpA, ClpP can degrade specific LacZ fusion proteins in vivo [5,6], as well as an endogenous *E. coli* protein, MazE [7], whereas, with ClpX, ClpP can degrade λ O protein [8,9], the P1 protein, Phd [10], and the *E. coli* sigma factor, RpoS [11], in vivo. Another ATPase of the Clp family, ClpY (HslU) appears to interact with and activate ClpQ (HslV) [8,12–15], a peptidase with no obvious evolutionary relatedness to ClpP. Specific substrates for ClpYQ in vivo have not been identified, although mutations in *clpY* and *clpQ* affect the levels of $\sigma^E$, an acute heat shock sigma factor [12].

We have been using negative staining electron microscopy and image analysis of purified Clp proteins in order to establish a structural basis for understanding the regulation and mechanism of action of Clp proteases. Previous studies demonstrated that ClpA is a symmetrical ring of six subunits [16], while ClpP is composed of two apposed rings of seven subunits each [16,17]. In the ClpAP complex, a hexameric ClpA ring is axially aligned on each face of the heptameric ClpP rings [16]. The mismatch in rotational symmetry between ClpA and ClpP dictates that individual subunits within ClpA rings make non-equivalent contacts with subunits of ClpP, and has been conjectured to be of functional significance, perhaps in facilitating rotational movement or processivity [16].

ClpAP displays a profound architectural similarity to the 26S proteasome, despite the lack of sequence similarity with proteasomal components. This similarity may imply a common mechanism of action. To explore the generality of this architecture, we have begun to examine the ClpYQ system. The heat shock *hslUV* operon of *E. coli* was identified by Chuang and Blattner [13] and shown by sequencing to encode two proteins, HslV (also called ClpQ [12]) and HslU (also called ClpY [8,12]). Because the latter is clearly related to the Clp family of ATPases, the names ClpY and ClpQ will be used in this paper. ClpY (subunit $M_r$ 50 kDa) has a Walker-type consensus sequence for ATP-binding proteins [18] and has extensive homology to ClpX [8,13]. ClpQ (subunit $M_r$ 19 kDa) has homology to the β-subunits of the 20S proteasome from Archaea and eukaryotes, including the catalytic amino terminal threonine residue [13,19]. The homology suggested that it might have a similar double-heptameric structure. Here, we show that ClpQ forms six-membered rather than seven-membered rings and that ClpY also contains six-membered rings.

## 2. Materials and methods

### 2.1. Materials

ATP, 5′-adenosine imidyldiphosphate (AMPPNP), and ATPγS were obtained from Sigma, IPTG from Bachem, and polyethylene imine from ICN. MonoQ, Q Sepharose, and Superose 6 were purchased from Pharmacia. The TSK250 gel filtration was obtained from BioRad. All other chemicals were reagent grade.

### 2.2. Cloning of clpQ and clpY

Double stranded DNA encoding either ClpQ or ClpY was prepared by amplification of *E. coli* chromosomal DNA using appropriate oligonucleotides derived from the published sequence of *hslUV* [13]. Amplified DNA was cloned into pVEX11, a derivative of a pET T7 expression vector [20], using the *Nde*I and *Bam*HI restriction sites. The sequences of *clpQ* and *clpY*, the fusion joints, and surrounding

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DNA were confirmed by automated sequencing using an Applied Biosystems 373 DNA sequencer.

*2.3. Protein purification*

*E. coli* BL21/DE3 *clpP*::Δ*kan* was transformed with pVEX11-*clpQ* and *E. coli* BL21/DE3 *clpX*::Δ*kan* was transformed with pVEX11-*clpY*. Cells were grown in Luria broth to an $A_{600}$ of 0.5, and production of T7 polymerase was induced by addition of 1 mM IPTG. After 2 h, cells were harvested by centrifugation and frozen at −70°C until used. To prepare crude extracts, frozen cells were thawed, suspended in 4 ml of 50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM DTT, and 10% (v/v) glycerol (buffer B) per g of cells, and broken by one pass through a French pressure cell at 20 000 psi. The extract was centrifuged at 30 000 × *g* for 45 min. A final concentration of 0.1% (w/v) polyethylene imine was added to the supernatant, and precipitated material was removed by centrifugation at 30 000 × *g* for 30 min.

For ClpQ, the supernatant solution was loaded onto a 2.6 × 20 cm column of Q Sepharose and proteins were eluted with a linear gradient of KCl in the same buffer. Fractions with ClpQ were identified by SDS-PAGE analysis and pooled. ClpQ was precipitated by the addition of cold acetone to 50% (v/v), collected by centrifugation, dissolved in buffer B, loaded onto a Mono Q HR16/10 column, and eluted with a linear gradient of KCl. ClpQ was precipitated from the pooled fractions with 50% (v/v) acetone, dissolved in 3 ml buffer B plus 0.2 M KCl, and run through a 2.3 × 60 cm TSK250 gel filtration column. Fractions containing ClpQ at ~95% purity were pooled and stored at −70°C.

For ClpY, the polyethylene imine supernatant was loaded onto a MonoQ HR16/10 column and eluted with KCl. ClpY was precipitated from the pooled fractions with 60% saturated ammonium sulfate, dissolved in buffer B plus 0.2 M KCl, and run over a TSK250 gel filtration column. ClpY in the major fractions was ~90% pure. Small aliquots (< 1 mg) of ClpY were further purified as needed by gel filtration on a Superose 6 HR10/30 column.

*2.4. Protein and enzymatic assays*

Protein concentrations were estimated from the $A_{280}$ of solutions and absorbance coefficients predicted from the amino acid sequences. Casein degradation assays were carried out as described previously [4].

*2.5. Electron microscopy and image averaging*

Specimens were prepared by placing 4 μl aliquots (20–50 μg/ml) protein in 50 mM Tris, pH 7.5, 0.2 M KCl, 1 mM EDTA, 1 mM DTT, 25 mM $MgCl_2$, 2 mM ATP, and 10% (v/v) glycerol on glow discharged carbon coated collodion covered grids. After 30 s excess buffer was blotted off. Aqueous 1% uranyl acetate was applied in 5–10 drops, excess stain was blotted off, and the grids were allowed to dry. Micrographs were recorded at × 50 000 magnification in a Zeiss 902 operating at 80 kV.

Micrographs were digitized with a Perkin-Elmer 1010G flatbed microdensitometer at 0.5 nm per pixel. General image processing and display operations were carried out using the PIC program [21] implemented on an Alpha workstation (Digital Equipment Corp., Maynard, MA). Particles were interactively extracted from digitized fields displayed on the workstation monitor, translationally aligned, analyzed for rotational symmetry, and subjected to correlation averaging as described [22].

## 3. Results

ClpQ and ClpY were overexpressed independently and purified. Based on visual examination of Coomassie blue stained gels, ClpQ was judged to be > 95% pure and ClpY was > 90% pure after the final steps. The sequences of the amino terminal 20 amino acids of purified ClpQ (beginning at Thr-2) and the first 17 amino acids of ClpY matched those predicted from the DNA sequences.

Table 1 shows that ClpQ was capable of degrading [$^3$H]*methyl*-casein to acid-soluble peptides in the presence of ClpY and ATP. ClpQ alone had a slight ability to degrade casein, and degradation activity was activated > 10-fold in the presence of excess ClpY. ATP-activated peptide degradation by ClpQ in the presence of ClpY was reported by the Chung and Goldberg laboratories [14,15].

Molecular weights of the purified proteins in the presence of ATP were estimated by gel filtration on Superose 6 (Fig. 1A). Apparent molecular weights were $2.2 \pm 0.2 \times 10^5$ for ClpQ (about 12 times the subunit molecular weight) and $3.5 \pm 0.4 \times 10^5$ for ClpY (about 7 times the subunit molecular weight). A similar molecular weight for ClpQ was recently reported by another laboratory, but their estimate for ClpY in the presence of ATP (~500 000) was significantly higher than ours [14,15].

When ClpQ was examined by negative staining electron microscopy, a preponderance of uniformly sized particles (diameter 10–11 nm) was observed. A number of molecules had a seemingly hexagonal outline. Micrographs of well stained fields (e.g. Fig. 2, left) were digitized and analyzed using an algorithm that detects rotational symmetries [22]. In each of two independent data sets, an unequivocal six-fold symmetry was detected, most strongly at a radius of ~5 nm from the center, around the outer rim of the particles (Table 2). No other orders of symmetry were found to be statistically significant. The molecular images were then enhanced using correlation averaging, which consistently depicted a regular six-fold symmetric particle, with a heavy accumulation of stain at the center (e.g. Fig. 2, left, inset). In the averaged image, six bright mass densities mark the vertices of a hexagonal particle, the edges of which have an inwardly scalloped contour.

ClpY in the presence of MgATP was visualized by electron microscopy after negative staining, revealing fields of rounded molecules of relatively uniform size (Fig. 2, right). Rotational symmetry analysis detected only six-fold symmetry (Table 2), which again was statistically significant only on the outer edges of the particles. Correlation averaging of three independent data sets consistently produced images of ClpY as a regular hexagonal particle with a maximum dimension of ~13 nm between opposite vertices (Fig. 2, right, inset). Compared with ClpQ, ClpY is somewhat larger, has non-scalloped edges, and has a less pronounced accumulation of stain in the center.

Because in the ClpAP complex association between ClpA and ClpP is stabilized by ATP, we anticipated that the affinity of ClpQ and ClpY for each other would be enhanced by an appropriate nucleotide. Fig. 1A shows that when a mixture of ClpY and ClpQ is run over a gel filtration column in the presence of MgATP, a species larger than both ClpY and ClpQ appeared. SDS-PAGE analysis of the column fractions showed that both ClpQ and ClpY were present in the higher molecular weight species (Fig. 1B). Thus, in the presence of nucleotide ClpY and ClpQ interact to form a complex isolatable by gel filtration, although evidence of some dissociation is apparent in the trailing shoulders. The $M_r$ of the ClpYQ

Table 1
ATP-dependent protein degradation by ClpYQ

| Addition | −ATP | +ATP |
|---|---|---|
| | (mg casein/h/mg) | |
| ClpY | < 0.1 | < 0.1 |
| ClpQ | 0.2 | 0.2 |
| ClpY+ClpQ | 0.2 | 6.5 |

Casein degradation was assayed using 0.2 μg ClpQ and 2 μg ClpY. Activity is expressed per mg of the limiting component, ClpQ.

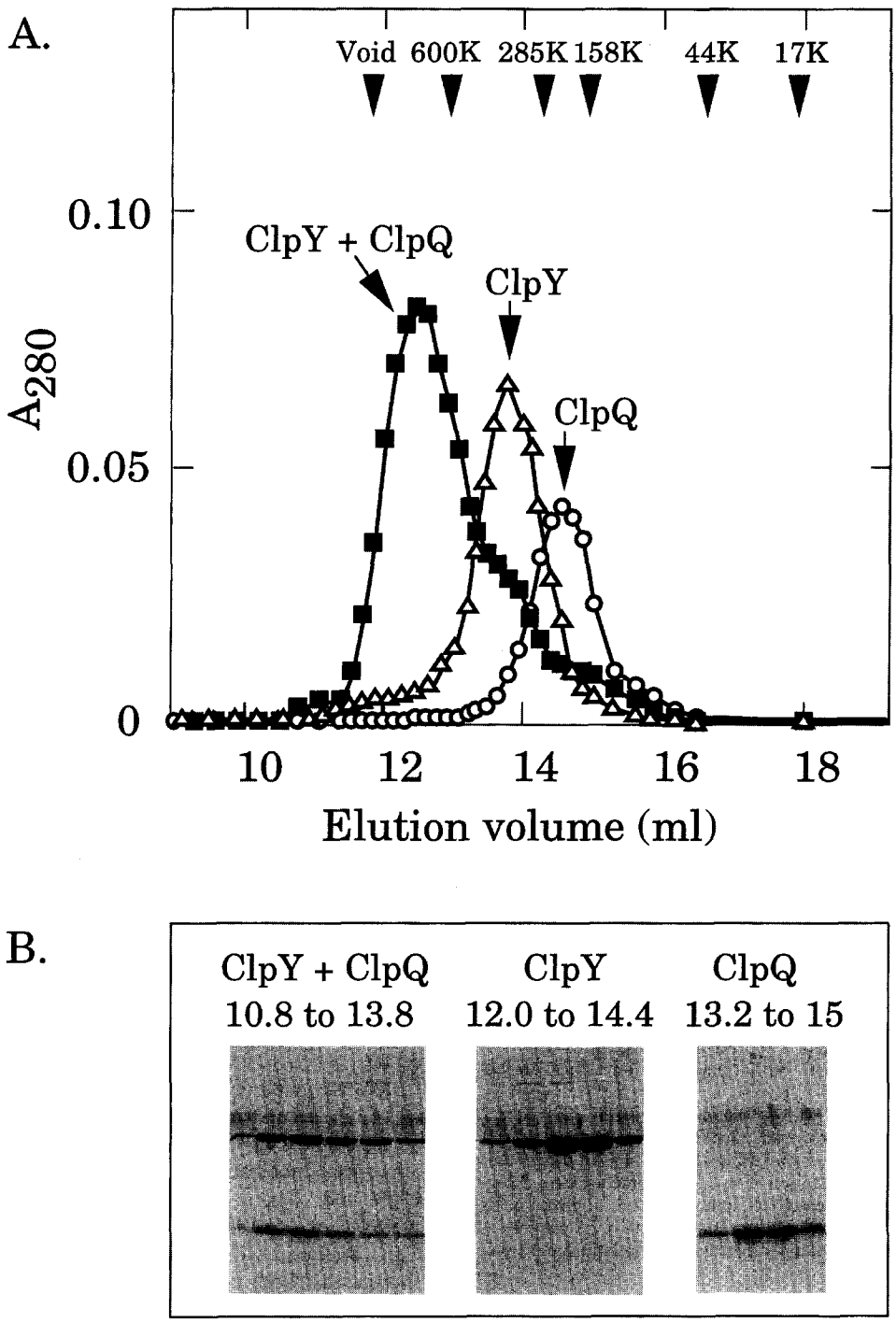


Fig. 1. Gel filtration of ClpQ, ClpY and the ClpYQ complex. A: A Superose 6 column was equilibrated with 50 mM Tris, pH 7.5, 0.2 M KCl, 1 mM EDTA, 1 mM DTT, 25 mM $MgCl_2$, 2 mM ATP, and 10% (v/v) glycerol. Protein samples in 200 μl of the above buffer were loaded and the column was run at 0.3 ml/min. (△) 300 μg ClpY; (○) 100 μg ClpQ; (■) a mixture of 300 μg ClpY and 100 μg ClpQ. The arrows indicate the elution position of standard proteins: thyroglobulin ($M_r$ 660 000), *E. coli* glutamine synthetase ($M_r$ 600 000), *E. coli* glutamate dehydrogenase ($M_r$ 285 000), aldolase ($M_r$ 160 000), ovalbumin ($M_r$ 45 000), and myoglobin ($M_r$ 17 000). A standard curve of log $M_r$ versus elution volume was used to estimate molecular weights of the proteins of interest with an accuracy of ±10%. B: SDS-PAGE analysis of proteins in the three gel filtration columns. Fractions of 0.6 ml were collected and 10 μl containing 1–2 μg of protein were mixed with SDS sample buffer, heated at 100°C, and run on 12% polyacrylamide gels in SDS. The numbers refer to the elution volumes for the different columns of the fractions chosen for analysis.

complex was > 600 000, consistent with a dodecamer of ClpQ interacting with at least one or possibly two hexamers of ClpY. Quantitation of the ClpY and ClpQ by scanning of the Coomassie stained SDS gels indicated that the complex had approximately equimolar amounts of both proteins (data not shown).

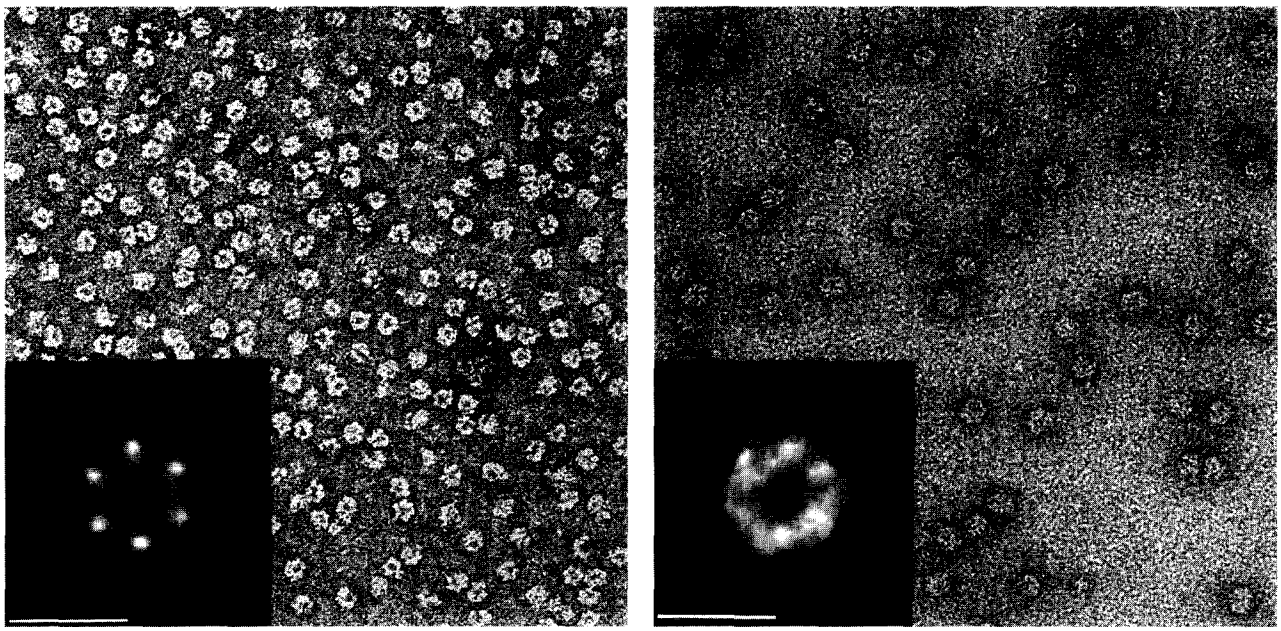

Fig. 2. Electron micrographs and average images of ClpQ and ClpY. Negatively stained ClpQ and ClpY were prepared as described in Section 2. Left: A typical field of wild-type ClpQ molecules; inset, averaged images of ClpQ viewed in en face projection. Right: Typical fields of wild-type ClpY molecules in the presence of ATP; inset, averaged images of ClpQ viewed in en face projection. Bar in insets is 50 nm.

Table 2
Image analysis of ClpY and ClpQ

| Sample | Total particles analyzed | Spectral[a] ratio product | Particles selected by OMO | Resolution[b] of averaged image (nm) | Dimensions[c] |
|---|---|---|---|---|---|
| A ClpY-1[d] | 96 | $6.0\times10^{28}$ | 77 | 2.8 | 13.5 |
| -2 | 200 | $1.3\times10^{10}$ | 194 | 2.0 | 13.6 |
| -3 | 201 | $2.0\times10^{23}$ | 197 | 2.2 | 13.5 |
| B ClpQ-1 | 106 | $8.3\times10^{14}$ | 103 | 3.2 | 11.0 |
| -2 | 139 | $4.5\times10^{70}$ | 109 | 2.6 | 12.5 |

[a]See Kocsis et al. [22]. The spectral ratio product is shown only for the radial zones in which these symmetries were detected most strongly (4.5–5.0 nm for ClpQ and 5.5–6.5 nm for ClpY).
[b]Resolution was determined using the spectral-signal-to-noise criterion [32].
[c]The maximum dimension of the particles taken as the distance between opposite vertices on the averaged hexagonal image.
[d]Numbers refer to independent data sets. For ClpY, data sets 1 and 2 were from micrographs of different fields of the same grid and data set 3 was

## 4. Discussion

ClpQ has six-fold rotational symmetry, implying that it is composed of one or more rings of six subunits. The subunit $M_r$ of 19 100 and approximate oligomeric $M_r$ of 220 000 are consistent with two such rings. Under our experimental conditions ClpY with bound ATP also forms an oligomer with six-fold rotational symmetry. Its $M_r$ ($\sim$350 000) together with subunit $M_r$ of 50 000 indicates that this oligomer is a single ring of six subunits. We have on occasion and under different conditions observed a component of seven-fold symmetric ClpY oligomers (preliminary experiments: data not shown)[2]; conditions that reproducibly form these apparent heptamers have not been defined. Micrographs of ClpY in the absence of nucleotide contained smaller less structurally defined particles, indicating less complete oligomerization (data not shown). Yoo et al. [15] observed lower molecular weight forms of ClpY upon gel filtration in the absence of ATP. However, under the conditions described here we reproducibly found ClpY with bound ATP to be predominantly hexameric.

A recurrent structural feature of oligomeric proteins involved in protein unfolding and degradation appears to be an arrangement of subunits to form a donut or torus with a channel in the center [16,23–28]. The super-complexes comprising the ATP-dependent proteases, e.g. the 26S proteasome [29] and ClpAP [16], are formed by axial stacking of two or more ring-shaped particles to form barrel-like structures with the two ATPase complexes related by relative rotation of 180° about a central proteolytic molecule possessing mirror symmetry. The structure appears designed simultaneously to shield the proteolytic active sites in the center, provide sites for interaction with substrates at the ends of the molecule, and allow substrates to be funneled through a central channel into the proteolytic active site cavity. From our data, the architecture of ClpYQ would be predicted to conform to this model.

[2]Evidence for six- and seven-fold symmetry in ClpY and for six-fold symmetry in ClpQ has been obtained independently by A.L. Goldberg, W. Baumeister, M. Rohrwild, H.C. Huang and A. Engel (Rohrwild, M. et al., submitted for publication).

Since seven-fold symmetry was first detected in the molecular chaperone, GroEL [23], this symmetry has been detected in numerous other oligomeric chaperones and proteases, including the accessory protein, GroES [30], the Archaea and the eukaryotic proteasomes, and *E. coli* ClpP [16]. However, other chaperones, such as Hsp104 [31] and ClpA [16], both of which have two ATPase domains, have six-fold symmetry. Our findings that ClpY, with a single ATPase domain, and ClpQ, with only a proteolytic domain, also possess six-fold symmetry clearly indicate that there is no universal structural imperative for seven-fold symmetry in oligomeric protein-remodeling enzymes.

A symmetry mismatch between the ATPase and the proteolytic core, as in ClpAP, is an intriguing feature of that protease. The presence of only six proteasome ATPase genes in yeast (D. Finley, personal communication) raises the possibility that a similar mismatch may exist in the 26S proteasome. However, ClpY and ClpQ, with six subunits per ring, presumably form a symmetric complex. Moreover, preliminary studies of ClpX (provided by T. Baker, Massachusetts Institute of Technology) show that it has the same seven-fold symmetry as its proteolytic partner, ClpP (unpublished data). Because functional complexes formed by interactions between rings of six (ClpYQ) or rings of seven (ClpXP) and between rings of six and seven (ClpAP) have been found, it follows that a symmetry mismatch is not an essential structural property of multi-component proteases.

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