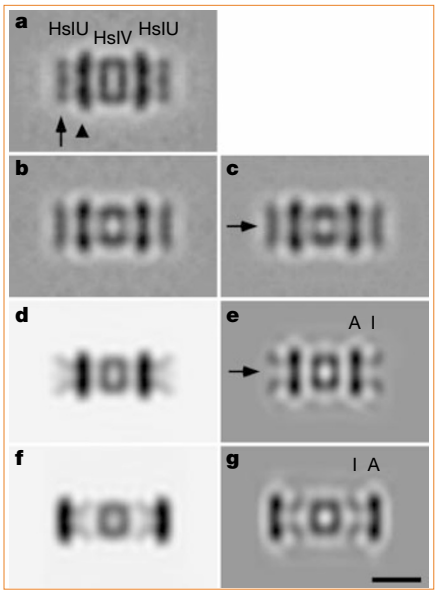

Figure 1 Averaged side-view projections of HslVU complexes. **a–c,** Electron micrographs of complexes formed in 50 mM Tris–HCl, pH 7.5, 0.2 M KCl, 10 mM $MgCl_2$ and 1 mM ATP, representing appropriate conditions for proteolytic activity. **a,** Negatively stained molecules (ATP-γS state; number of particles, $N = 65$; resolution, 32 Å). Note that the proximal ring of HslU (arrowhead) is wider and more dense than the outer ring (arrow); **b,** frozen-hydrated molecules (ATP-γS state; $N = 250$; resolution, 33 Å); **c,** frozen-hydrated molecules in the AMP-PNP state, where AMP-PNP is an inactive ATP analogue ($N = 400$; resolution, 33 Å). **d–g**, Side-view projections calculated[6] from the crystal structure[2] but limited to 30 Å resolution. Projections corresponding to different rotational settings of the complex around the axis were averaged to give a cylindrically averaged side view, as in the electron micrographs (EMs). In **d** and **e**, HslU is in the opposite orientation from the one in the crystal structure, whereas in **f** and **g** this corresponds to the published orientation[2]. Projections shown in **e** and **g** were created by applying a phase-contrast transfer function (CTF; corresponding to 2.0 μm underfocus) to images in **d** and **f**, and so are more comparable to the cryo-EMs. With or without CTF correction, it is evident that the wider, denser ring, corresponding to the ATPase domains of HslU, is adjacent to HslV. Arrows in **e** and **c** mark the axial density that is missing in **e** but present in **b** and **c**, which we attribute to residues 175 to 209. In **e** and **g**, I denotes the I-domain ring, and A denotes the ATPase-domain ring. Scale bar, 100 Å.

the images are inconsistent with a side-view projection (Fig. 1f) generated from the published coordinates of HslVU[2] and limited to the same resolution[6].

For closer comparison, we subjected this image computationally to phase-contrast effects (Fig. 1g), simulating those of the cryo-electron microscopy images. Conversely, an excellent match was obtained with similarly generated projections (Fig. 1d, e) in which HslU was inverted (compare Fig.1c, e). We conclude that the I-domains are exposed on the distal surfaces of the HslVU complex, and the opposite face of the HslU ring binds to HslV. Despite good overall agreement with the results from cryo-electron microscopy, the calculated reprojection shows the central part of the distal ring of HslU as relatively depleted in density (arrows in Fig.1c, e). We infer that the additional density in the electron microscopy images represents six copies of residues 175–209 which were not seen in Bochtler *et al.*'s crystal structure[2].

HslU belongs to the AAA superfamily[7] of ATPases, as do the ATPases of the 26S proteasome, and ClpA and ClpX of *E. coli*, which both partner the protease ClpP (ref. 1). As demonstrated for ClpA[8] and ClpX[9], all such ATPases are likely to have protein 'unfoldase' activity. Processive degradation is carried out by fully assembled holoenzymes[10] and requires the coordinated activity of multiple sites. The geometry of interaction between the ATPase and proteinase rings is crucial in specifying the positions of the sites at which substrates bind, where they are unfolded, and the path along which they translocate into the digestion chamber inside the protease.

Our model assigns the I-domains to the distal surfaces of the HslVU complex, in an exposed position that would be suitable for substrate binding. This proposition is consistent with data showing protein substrates binding to the distal surfaces of both ClpXP[11] and ClpAP (T.I. *et al.*, manuscript submitted). In this revised model, residues in the ATPase domain of HslU, which includes the carboxy-terminal sensor-2 domain[7], are responsible for binding to HslV.

Takashi Ishikawa, Michael R. Maurizi*, David Belnap, Alasdair C. Steven

*Laboratory of Structural Biology, National Institute of Arthritis, Musculoskeletal and Skin Diseases and *Laboratory of Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892, USA*

e-mail : steven@calvin.niams.nih.gov

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Bochtler et al. *reply* — The central issue raised by Ishikawa *et al.* is that of the configuration of the productive HslVU complex. We note that complexes of HslV and HslU in *E. coli* are labile and unstable under many conditions[1]. The original electron microscopy (EM) images of Rohrwild *et al.*[2] appear to show free HslV, free HslU and HslV–HslU complex particles. To explain the discrepancy between the HslV–HslU arrangement in our co-crystals and their negatively stained EM data, we suggested that there might have been a collapse of the fragile I-domain structure in the EM preparations, or a reversal in the orientation of the HslU rings[1]. Ishikawa *et al.* interpret their results from cryo-EM at 30 Å resolution in this latter way, using our crystal data of the components. Although these preparations preserve the native structure better than the negatively stained ones, our HslV–HslU samples are also active under crystallization conditions[3].

We have attempted to distinguish between the two docking modes (I-domains distal or proximal to HslV) in mutagenesis experiments involving more than two dozen mutants[3]. We disrupted putative contact sites to HslV in the I-domain of HslU (Fig. 3b in ref. 1) and on its opposite face and find none of these mutations has any effect on peptide hydrolysis or on casein degradation. This suggests either that no precise complex is required, or that both modes of docking are feasible. In contrast, degradation of the physiological substrate fusion protein MBP–SulA is affected by mutations both in the I-domain as well as those involving the opposite side of HslU. Small-angle X-ray scattering data and a crystal structure of the *Haemophilus influenzae* HslVU complex are also consistent with the EM docking mode[4].

Matthias Bochtler*, Claudia Hartmann, Hyun Kyu Song, Ravishankar Ramachandran, Robert Huber

Abteilung Strukturforschung, Max-Planck-Institut für Biochemie, Am Klopferspitz 18a, Planegg-Martinsried D-82152, Germany

e-mail: huber@biochem.mpg.de

**International Institute of Molecular and Cell Biology, ul. Ks. J. Trojdena 4, 02-109 Warszawa, Poland*

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Addendum

A new model for protein stereospecificity

A. D. Mesecar & D. E. Koshland Jr

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To clarify possible misunderstanding over the term "new" in this communication, we meant our new model replaces the old Ogston model, as this is incompatible with our X-ray crystallographic data. We did not intend "new" in this context to mean that nobody had ever questioned the Ogston model before (for example, see refs 1–5).

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Editorial note: As Brief Communications do not allow space for conventional introductions, we asked the authors for this addendum to clarify a possible misinterpretation.