Supporting information for Ramachandran *et al*. (May 21, 2001) *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas. 102188799.

Experimental Procedures

Cloning, Expression, and Purification. Standard PCR techniques were used to generate mutants, which were subsequently ligated into pET12b and the resultant constructs transformed into Bl21(DE3) cells for expression. The mutants were purified as previously described for wild-type HslV (1) and HslU (2) enzymes. Each mutagenesis was confirmed by DNA sequencing and/or electron spray ionization mass spectroscopic analyses. The state of oligomerization was estimated by gel filtration and comparison with the elution profile of the wild-type protein. All mutants exhibited similar elution profiles as the wild-type enzyme unless stated otherwise.

Assays. In the course of our experiments, we noticed that contaminating amounts of ADP in our ATP stocks could interfere with HslVU activity and introduce substantial variability into results of the chromogenic peptide and caseinolytic assays. Use of pyruvate kinase and phosphoenolpyruvate to regenerate ATP solved this problem. In our assays, we used the nonhydrolyzable ATP analogue, ATP-γS, for the peptide and caseinolytic assays. For the MBP-SulA substrate, we used ATP because degradation of this substrate depends on ATP hydrolysis.

Peptide hydrolysis was assayed using the chromogenic peptide, carbobenzoxy-Gly-Gly-Leu-7-amido-4-methylcoumarin (Z-Gly-Gly-Leu-AMC; Bachem) as a substrate as reported (3) with 1 µg of HslV and 2.5 µg of HslU.

FITC-labelled casein (4) was used as a model flexible protein substrate. The degradation of FITC--casein was measured using HPLC. Enzyme samples (6 µg of HslV/15 µg of HslU) in buffer U (50 mM Tris•HCl, pH = 7.5, 5 mM $MgCl_2$) were incubated for 45 min with 2 mM adenosine 5′-O-(3-thiotriphosphate) (ATP-γS) and 1 mM FITC--casein. The reaction was stopped by the addition of calpain inhibitor I (Acetyl-Leu-Leu-Norleucinal). Alternatively, the caseinolytic activity was assayed using radio-labelled ^{14}C-methyl casein (5) as reported (2).

The MBP-SulA assay was carried out as reported (6-8) with minor modifications.

The peptide activation assays against FITC--casein and MBP-SulA were performed as above, except that HslU was replaced by 200 µM of the respective peptides. In the assay against Z-Gly-Gly-Leu-AMC, varying concentrations (0.25-100 µM) of the respective ++activator peptides were used. We point out that our dose--response curves show the biological activity of the activator peptides but do not allow derivation of a precise K_d, because endpoints and not rates of hydrolysis of the chromogenic substrate have been measured and because, in the regime of low peptide activator concentrations, activator peptide is not present in sufficient amounts to saturate all binding sites on HslV.

Fluorescence Spectroscopy. Fluorescence measurements were carried out by using a Perkin--Elmer instrument (Model LS50B). The dansylated peptide activator (Dansyl-EDLSRFIL) was added in aliquots of 1.5/2.5 µl from a 1,250 µM stock solution to a

1.5-ml solution of HslV (monomeric concentration of 26 μM) kept in a microcuvette equipped with a magnetic stirring bar, and the fluorescence spectra were recorded. For experiments involving inhibited HslV, the protein was incubated with calpain inhibitor I for 1 hr before the measurements. A similar experiment was performed where the peptide was added only to the buffer in which the protein was present, namely 20 mM Tris/300 mM NaCl/0.002 % NaN_3. We found enhancement in fluorescence in the presence of protein compared to the experiment in its absence. The excitation and emission slit widths were kept at 2.5 and 5.0 nm, respectively. Emission and excitation wavelengths used (335 and 533 nm, respectively) were determined by a spectral scan of a solution of the dansylated peptide in the absence and presence of protein. ΔF_{533} values were obtained by correcting the signal from each spectrum of the complex in the fluorescence titration experiment at 533 nm for the contribution of the protein and peptide, respectively. The dissociation constant (K_d) was calculated as reported earlier (9) by fitting it together with ΔF_{max} using an equation derived from the law of mass action to the ΔF_{533} values:

$$\Delta F_{533} = (\Delta F_{max} /[L]_t n)[A - \mathrm{sqrt}(A^2 - [L]_t n[R]_t)],$$

where $A = ([R]_t + [L]_t n + K_d)/2$, $[L]_t$ = total concentration of the peptide, $[R]_t$ = total concentration of the protein, $\Delta F_{max} = \Delta F_{533}$ of the complex at infinite ligand concentration, n = number of binding sites per monomer, K_d = dissociation constant of the HslV–Dansyl-EDLSRFIL complex.

References:

1. Bochtler, M., Ditzel, L., Groll, M. & Huber, R. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 6070-6074.

2. Bochtler, M., Hartmann, C., Song, H. K., Bourenkov, G., Bartunik, H. D. & Huber R. (2000) *Nature (London)* **403,** 800-805.

3. Rohrwild, M., Coux, O., Huang, H. C., Moerschell, R. P., Yoo, S. J., Seol, J. H., Chung, C. H. & Goldberg, A. L. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 5808-5813.

4. Twining, S. S. (1984) *Anal. Biochem.* **143,** 30-34.

5. Driscoll, J. & Goldberg, A. L. (1990) *J. Biol. Chem.* **265,** 4789-4792.

6. Song, H. K., Hartmann, C., Ravishankar, R., Bochtler, M., Behrendt, R., Moroder, L. & Huber, R. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 14103-14108.

7. Higashitani, A., Ishii, Y., Kato, Y. & Horiuchi, K. (1997) *Mol. Gen. Genet.* **254,** 351-357.

8. Seong, I. S., Oh, J. Y., Yoo, S. J., Seol, J. H. & Chung, C. H. (1999) *FEBS Lett.* **456,** 211-214.

9. Sondermann, P., Jacob, U., Kutscher, C. & Frey, J. (1999) *Biochemistry* **38,** 8469-8477.