

Quantitative NMR spectroscopy of supramolecular complexes: Dynamic side pores in ClpP are important for product release

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The highly conserved, 300-kDa cylindrical protease ClpP is an important component of the cellular protein quality machinery. It consists of 14 subunits arranged into two heptameric rings that enclose a large chamber containing the protease active sites. ClpP associates with ClpX and ClpA ATPases that unfold and translocate substrates into the protease catalytic chamber through axial pores located at both ends of the ClpP cylinder. Although the pathway of substrate delivery is well established, the pathway of product release is unknown. Here, we use recently developed transverse relaxation optimized spectroscopy (TROSY) of methyl groups to show that the interface between the heptameric rings exchanges between two structurally distinct conformations. The conformational exchange process has been quantified by magnetization exchange and methyl TROSY relaxation dispersion experiments recorded between 0.5°C and 40°C, so that the thermodynamic properties for the transition could be obtained. Restriction of the observed motional freedom in ClpP through the introduction of a cysteine linkage results in a protease where substrate release becomes significantly slowed relative to the rate observed in the reduced enzyme, suggesting that the observed motions lead to the formation of transient side pores that may play an important role in product release.

methyl transverse relaxation optimized spectroscopy | protein dynamics | ClpP protease

ClpP (1) is a representative member of the family of cylindrical self-compartmentalizing proteases (2, 3) that include the bacterial protein HslV (4) as well as the archaeal and eukaryotic 20S proteasomes (5, 6). The proteolytic active sites of these proteases are located in an enclosed catalytic chamber separated from the cellular milieu. Proteins targeted for degradation are recognized by AAA⁺ chaperones that unfold and translocate substrates in an ATP-dependent manner into the lumen of the protease. In the case of ClpP (Fig. 1 *a–c*), it is well established that ClpX and ClpA deliver substrates through axial pores into the protease catalytic chamber for subsequent degradation (Fig. 1*a*) (1, 7, 8). In contrast, the pathway of product release remains controversial. For ClpP (1, 9, 10) and for the proteasome (11), it has been proposed that the entrance pores might also function as product exit sites. However, this proposal is problematic, because it requires the chaperones, which can bind to both ends of the protease simultaneously, to interrupt translocation to allow product release (12).

Recently, it has been shown that the ClpP handle region that forms the main interface between two ClpP rings (Fig. 1*b*) can be deleted without disrupting the oligomeric state of the protein. Furthermore, the x-ray structure of an A153P mutation of *Streptococcus pneumoniae* ClpP (13) shows that the mutation causes two turns of helix E to become unstructured. Nevertheless, the mutant protein remains a double heptamer, which suggests a high plasticity for the handle region (Fig. 1*d*). Here, we used recently developed NMR methodology (14, 15) and isotope labeling schemes (16) involving the use of methyl group probes to establish that the handle region is indeed dynamic, with

an equilibrium between two distinct conformational states. Despite the high molecular mass of the complex (>300 kDa) and the low temperature used for many of the studies (0.5°C), it is, nevertheless, possible to obtain quantitative measures of both the kinetics and the thermodynamics of the exchange process. Where possible, results from one type of NMR experiment have been corroborated with those from a second, establishing that the NMR approaches are robust, even in applications involving supramolecular structures. Biochemical assays that are complementary to the NMR study suggest a possible role for the dynamics in product release from ClpP. The present investigation shows that current NMR techniques are now at a stage where insight into biological function in even very large systems can be obtained.

Materials and Methods

Protein Expression and Purification. A pET9a vector overexpressing untagged WT *Escherichia coli* ClpP was a gift from John Flanagan (Brookhaven National Laboratory, Upton, NY). ClpP A153C, ClpP S111A A153C, ClpP Δ N (with a deletion of the 21 N-terminal residues, including the 14-residue prosequence), and ClpP Δ N S111A A153C were cloned into pET3a vectors without the prosequence and purified as described in ref. 17. ClpP I149V and I151L mutants were cloned into a modified pET-15b vector, which introduces an N-terminal histidine tag followed by a tobacco etch virus protease-recognition sequence. Point mutations were introduced with the QuikChange system (Stratagene) according to the manufacturer's protocol. U-[¹⁵N,²H], Ile δ 1-[¹³C,¹H] ClpP mutant samples were obtained by protein overexpression of the corresponding gene in BL21(DE3) cells in 250 ml of 100% D₂O minimal medium, as described in ref. 18. Purification was achieved by using Ni affinity chromatography, cleavage of the histidine tag, and size-exclusion chromatography. NMR experiments were performed on samples with protein monomer concentrations of 0.3–0.5 mM in 100% D₂O containing 50 mM potassium phosphate buffer (pH 6.8, uncorrected), 0.1 mM EDTA, 1 mM DTT, and 0.03% NaN₃. GFP-SsrA degradation assays, *N*-succinyl-Leu-Tyr-7-amido-4-methylcoumarin (Suc-LY-AMC) (Sigma) cleavage assays, and CD experiments were carried out as described in ref. 13.

Denaturing, Nonreducing SDS/PAGE. Oxidized forms of the ClpP A153C mutants were readily obtained in the absence of reducing agent. The oxidized forms were reduced by incubating with 10 mM DTT for 1 h at 37°C. To visualize the disulfide-linked forms of the ClpP A153C mutants on nonreducing SDS/PAGE gels,

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Abbreviations: TROSY, transverse relaxation optimized spectroscopy; Suc-LY-AMC, *N*-succinyl-Leu-Tyr-7-amido-4-methylcoumarin.

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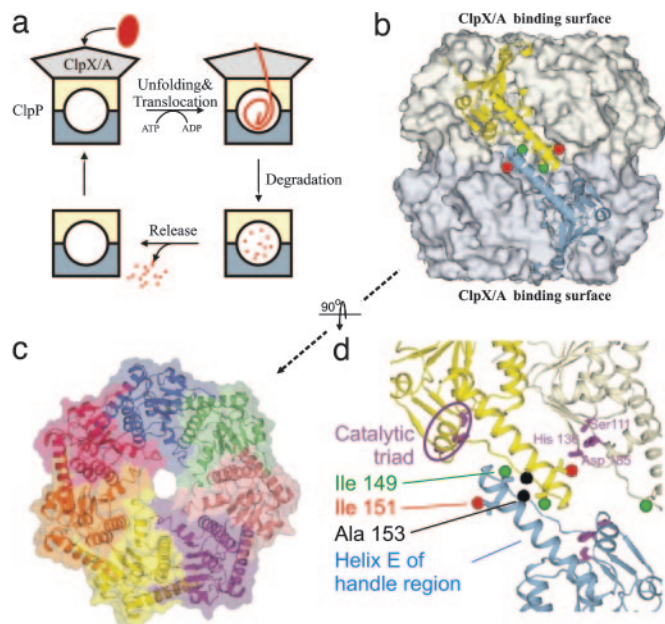


Fig. 1. ClpP structure and function. (a) Schematic diagram illustrating the mechanism of function of the ClpX/A–ClpP complex. ClpX/A unfolds and translocates substrate proteins into the ClpP proteolytic chamber for degradation in an ATP-dependent manner (8). ClpX/A molecules can bind ClpP from the top and bottom simultaneously. (b and c) *E. coli* ClpP side and top views. The axial pores are lined with the N-terminal residues of ClpP (13, 29) that are invisible in the *E. coli* x-ray structure (1). (d) Detailed view of the ClpP handle region. Residues of the catalytic triad are circled. The C δ 1 atoms of I149 and I151 are indicated by green and red spheres, respectively. The position of the A153C mutation is highlighted in black. Figures displaying molecular structures were prepared with PYMOL (www.pymol.org).

samples were incubated for 30 min with 55 mM iodoacetamide (Sigma) at 37°C, followed by additional heating at 95°C for 5 min.

Retention of Suc-LY-AMC by ClpP Cysteine Mutants. A ClpP cysteine mutant (20 μ M) was incubated with 4.4 mM fluorogenic peptide, Suc-LY-AMC, in a total volume of 200 μ l of buffer (25 mM Tris-HCl, pH 7.5/200 mM NaCl/10% glycerol) for 30 min at room temperature in the dark. The mixture was then applied on a calibrated Superdex 200 HR 10/30 column, and 1.0-ml fractions were collected over two column volumes. The fluorescence of the samples was measured on a Fluorolog 3-222 spectrofluorometer (Jobin Yvon, Longjumeau, France) using an excitation wavelength of 325 nm, and the emission scans were collected from 360 to 440 nm. Protein contents of the fractions were visualized on SDS/PAGE gels.

NMR Spectroscopy. NMR spectra were recorded with 600 and 800 MHz Varian Inova spectrometers equipped with cyro (600 MHz) or room-temperature (800 MHz) pulsed-field gradient triple resonance probes. All data were processed with the NMRPIPE/NMRDRAW suite of programs (19). Typical acquisition times for carbon chemical shift evolution were 20 ms, with the center of the carbon spectrum at 12.0 ppm. ^1H and ^{13}C chemical shift assignments are referenced against DSS (2,2-dimethyl-2-silapentane-5-sulfonate). Longitudinal magnetization transfer experiments were recorded as a series of 3D data sets with mixing times, t_{MIX} , between 0 and 60 ms. Briefly, magnetization is transferred from ^1H to ^{13}C , and the carbon chemical shift of the starting state is recorded (t_1). Subsequently, two-state longitudinal order is created that is allowed to exchange during a mixing time, followed by ^{13}C shift evolution (t_2) and transfer to ^1H for detection (t_3). The methyl transverse relaxation optimized

spectroscopy (TROSY) principle (14) was exploited throughout the experiment. In this manner, correlations at (ω_{Ca} , ω_{Cb} , ω_{Ha}) and (ω_{Ca} , ω_{Cb} , ω_{Hb}) are obtained, corresponding to autopeaks and cross-peaks, respectively, where the two states are denoted by *a* or *b* and correspond to either S or F (see Results and Discussion). The data were fitted according to $I_{\text{cross}} = A p_a p_b \exp(-\rho t_{\text{MIX}}) [1 - \exp(-k_{\text{ex}} t_{\text{MIX}})]$ and $I_{\text{auto}} = B \exp(-\rho t_{\text{MIX}}) [p_a^2 + p_b^2 \exp(-k_{\text{ex}} t_{\text{MIX}})]$, where $I_{\text{cross/auto}}$ is the intensity of the cross-/autopeak, $p_{a/b}$ is the population of state *a/b*, ρ is the longitudinal relaxation rate of the two-state order, $H_z C_z$ (where X_z is the Z magnetization of spin $X = \{^1\text{H}, ^{13}\text{C}\}$), and k_{ex} is the exchange rate. Intensities were scaled according to the intensity of the corresponding autopeak at the shortest mixing time. The factors A and B take into account differential relaxation between the two states during the delays in the pulse sequence. Autorelaxation rates ρ were determined separately by using a 2D version of the exchange experiment where the first carbon evolution period was omitted. In the expressions for $I_{\text{cross/auto}}$ above, it is assumed that the autorelaxation rates for the two states are the same (i.e., $\rho_a = \rho_b$ for each residue, although they may differ for different residues). Indeed, measurements of ρ_a , ρ_b differ by <10% for either I149 or I151. In any event, the assumption of $\rho_a = \rho_b$ has little influence on the extracted rates or populations because $\rho_a, \rho_b \ll \rho_a k_{\text{ex}}, \rho_b k_{\text{ex}}$.

Methyl TROSY relaxation dispersion experiments (20) were recorded as a series of 2D data sets using constant-time relaxation periods of 15 ms (0.5°C), 10 ms (600 MHz, 35°C, 40°C), or 7 ms (800 MHz, 35°C, 40°C) and CPMG (Carr–Purcell–Meiboom–Gill) frequencies ranging from 66.7 to 1,000 Hz. $R_{2,\text{eff}}$, the effective transverse relaxation rate, is calculated according to $(-1/T) \ln(I/I_0)$, where I (or I_0) is the intensity of a correlation in the presence (or absence) of a constant-time relaxation interval of duration T , during which a variable number of ^{13}C 180° pulses are applied (20) and $\nu_{\text{CPMG}} = 1/(2\delta)$, where δ is the time between successive pulses. The dispersion data were fitted numerically as described in ref. 20. Chemical shift differences were initially estimated from spectra recorded at 0.5°C and then optimized based on the extracted exchange parameters from fits of the dispersion data. This optimization is accomplished in an iterative manner whereby the fitted exchange parameters are used, along with estimated shift differences, to obtain the true shift differences from the eigenvalues of the free precession evolution matrix. Errors in all exchange rates were estimated by using Monte Carlo methods based on the uncertainties in peak intensities.

Results and Discussion

NMR Resonance Assignment. The ClpP tetradecameric complex represents one of the largest systems studied quantitatively by using NMR methodologies. Unlike the case for NMR studies of the 900-kDa GroEL–GroES complex (21), ^{15}N – ^1H correlation maps (22) for ClpP were of low quality, likely reflecting the large amount of dynamics in the oligomer (see below). By contrast, methyl TROSY (14) spectra of highly deuterated, Ile δ 1-methyl protonated ClpP were of high quality. At least 12 of the expected 16 correlations are observed, even at temperatures as low as 0.5°C, where the rotational correlation time of the complex is estimated to be >400 ns (14). Ile probes are particularly useful in the present case, because the handle region contains two Ile residues (I149 and I151; Fig. 1 *b* and *d*) that can be used to report on the dynamics of this region.

Before a detailed NMR study, the chemical shifts of the isoleucines of interest must be assigned. The large size of ClpP precludes the use of traditional assignment strategies based on multidimensional experiments, and assignments were obtained by a mutational approach. For example, I149 and I151 were replaced by Val and Leu, respectively (Fig. 2*a*). Interestingly, in correlation spectra recorded at 0.5°C, δ 1-methyls of both residues give rise to sets of two resonances (Fig. 2*a*), consistent with both isoleucines adopting a pair of distinct conformations.

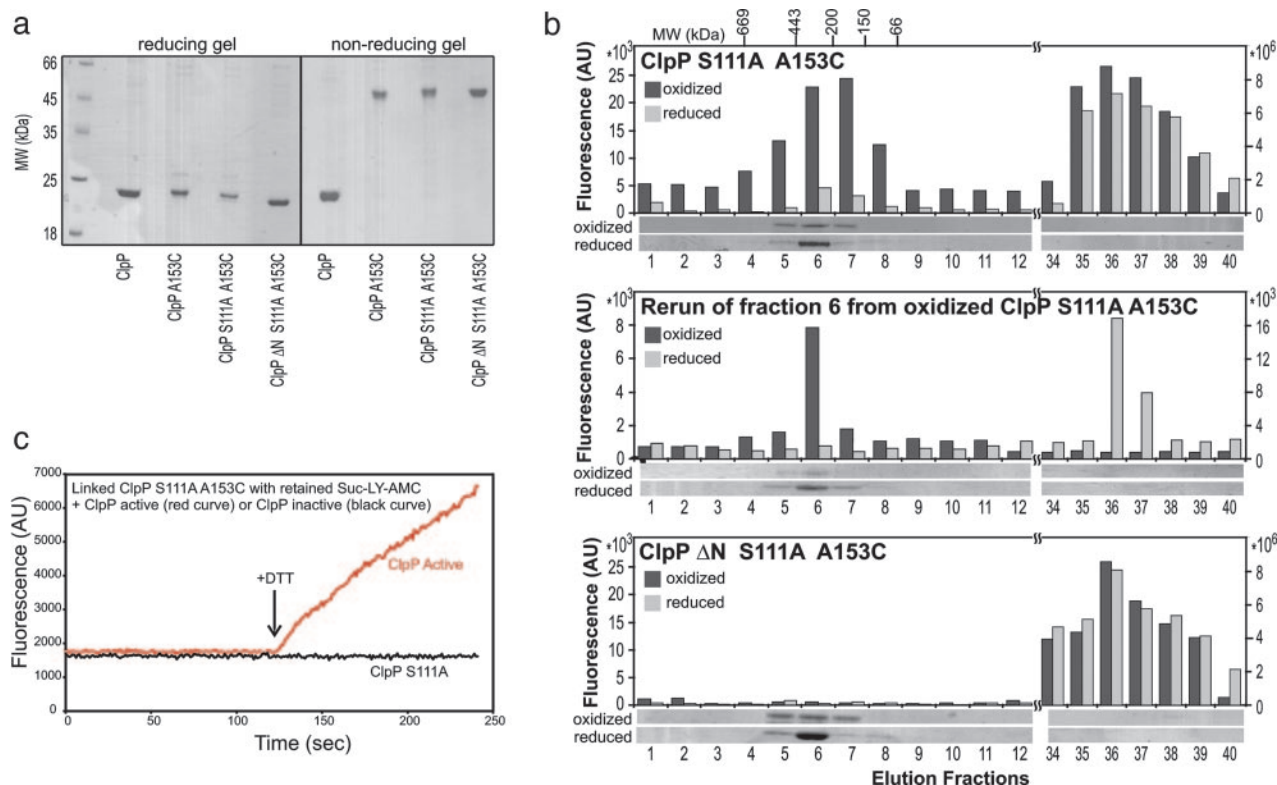


Fig. 4. The flexible handle regions affect product release. (a) SDS/PAGE analysis of ClpP WT and ClpP A153C mutants. Under reducing, denaturing conditions, all proteins migrate as monomers. Under nonreducing, denaturing conditions, the Cys mutants migrate as dimers, linked by disulfides at Cys-153. The position of the disulfide link and the molecular weight under nonreducing denaturing conditions has been verified by mass spectrometry (data not shown). (b) Size-exclusion chromatography profiles (using a calibrated Superdex 200 column) of ClpP incubated with Suc-LY-AMC fluorescent peptide. The fluorescence of each fraction (probing the presence/absence of peptide) was measured (bar graphs), and the protein content was visualized by SDS/PAGE analysis (below bar graphs). (Top) Under oxidizing conditions, the peptide is retained in the linked form of ClpP S111A A153C. Excess peptide in the incubation mixture that is not in the lumen of the protease elutes at $1.5 \times$ the column volume because the free peptide interacts weakly with the column. No peptide is retained under reducing conditions. (Middle) Fraction 6 from the experiment under oxidizing conditions in Top was rerun on the column. The peptide remains in the lumen of the protease under oxidizing conditions and is released under reducing conditions. (Bottom) ClpP Δ N S111A A153C does not retain the peptide, likely reflecting that deletion increases the size of the axial pores. (c) Inactive, oxidized ClpP (S111A A153C) was incubated with the fluorescent model peptide Suc-LY-AMC, and excess peptide was dialyzed out. At time $t = 0$, either active ClpP (red line) or inactive ClpP (black line) was added, and the fluorescence profile was recorded. At time $t = 120$ s, DTT was added to the mixture, resulting in a release of the peptide from the cysteine-linked ClpP. The released peptide was readily cleaved by the added active form of ClpP, resulting in an increase of the fluorescence (red line). The added inactive ClpP cannot cleave the model peptide and, as a result, the fluorescence does not change (black line).

interconverting states (23) (Fig. 3a; see also Fig. 6a, which is published as supporting information on the PNAS web site). When both isoleucines are fitted individually, very similar exchange parameters are extracted, consistent with a common exchange process, as suggested by the similar temperature dependence of the corresponding peak intensities. A global fit yields an exchange rate of $k_{\text{ex}} = k_{\text{F} \rightarrow \text{S}} + k_{\text{S} \rightarrow \text{F}} = 60.8 (7.0) \text{ s}^{-1}$ and similar populations, $p_{\text{F}} = 0.58 (0.03)$, $p_{\text{S}} = 0.42 (0.03)$, at 0.5°C .

The exchange process can be quantified in an independent way by using methyl TROSY relaxation dispersion measurements (20) (Fig. 3b). Here, the effective line width ($R_{2,\text{eff}}/\pi$) of correlations is measured as a function of the applied field strength, ν_{CPMG} , and the dependence is sensitive to exchange rates, populations of interconverting states, and both ^1H and ^{13}C chemical shift differences between states that can be measured directly from spectra recorded at 0.5°C (Fig. 2a, WT). As observed for the longitudinal exchange experiments, exchange parameters extracted for individual residues are very similar, and $k_{\text{ex}} = 67.5 (2.5) \text{ s}^{-1}$, $p_{\text{F}} = p_{\text{S}} = 0.5 (0.02)$ were obtained from a global fit of the dispersion data. The utility of methyl TROSY techniques to access dynamic information on very large protein

complexes can be appreciated from the similar exchange values obtained by means of the two independent methods.

The temperature dependence of the exchange parameters has been obtained by measuring longitudinal exchange between 0.5°C and 7°C (Fig. 3c). At higher temperatures, exchange contributions to the line widths of the correlations for I149 and I151 prevent the quantification of peak intensities. Over this limited temperature range, a clear increase in exchange rates is observed without a significant shift in populations (Fig. 6). At temperatures $>35^\circ\text{C}$, the resonances of both isoleucines reappear at chemical shifts that are average between states F and S. Although the absence of discrete states precludes analysis by means of longitudinal exchange, extraction of exchange parameters is possible using dispersion measurements and the knowledge of the chemical shift differences observed at low temperature. The dispersion profiles fit very well to an increased exchange rate predicted from the extrapolation of the low-temperature data and to populations close to 1:1 (Fig. 3c Inset). Using measured exchange rates from 0.5°C to 40°C and transition state theory, values for the activation enthalpy [$\Delta H^\ddagger = 13.1 (0.6) \text{ kcal} \cdot \text{mol}^{-1}$] and entropy [$\Delta S^\ddagger = -3.6 (2.3) \text{ cal} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$] for the F,S interconversion were calculated. Interestingly, the $\Delta H^\ddagger$ value obtained is similar to that reported previously for a localized conformational rearrangement in a cavity mutant of T4 lysozyme

In conclusion, the present study demonstrates the use of NMR experiments to establish that the handle regions connecting the ClpP rings are highly dynamic and to characterize the kinetic and thermodynamic properties of the dynamic process. Biochemical approaches have been used, showing that cross-links between the E helices in the handle region significantly increase the retention time of peptide in the lumen of the enzyme relative to a reduced protein. Together, these results are consistent with the importance of dynamics in this region for the establishment of equatorial exit pores for the release of ClpP products. Exit of substrate through side pores provides a mechanism for the spatial separation of substrate entrance and product release when ClpX/A is bound to both axial pores simultaneously (Fig. 5), which may be an important component for the efficient and processive operation of the protease, allowing for continuous ClpX/A-mediated translocation of substrate into the proteolytic chamber through both axial pores at the same time (12, 28). The similarities in the structure and subunit arrangements between the members of cylindrical self-compartmentalizing proteases,

and the fact that they all associate with ATPases that unfold and translocate substrates into an enclosed catalytic chamber for proteolysis (2, 3), suggest that the proposed model for peptide exit might be conserved. This work highlights the utility of methyl TROSY NMR spectroscopy to quantify site-specific dynamics in supramolecular structures and the power of a combined NMR-biochemistry approach for the study of biomolecular function.

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