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.

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 ΔN (with a deletion of the 21 N-terminal residues, including the 14-residue prosequence), and ClpP ΔN 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,13C]H, H[1-15C]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,
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 mM) was incubated with 4.4 mM fluorogenic peptide, Suc-LY-AMC, in a total volume of 200 µl 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 to the top and bottom simultaneously. (invisible in the experiments were recorded as a series of 3D data sets with mixing time, followed by 13C shift evolution (T), where I (t) 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 13C 180° pulses are applied (20) and δCPMG = 1/(2δ), 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), 15N-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, Ile3 molecules can bind ClpP from the ClpP side and top views. (b) ClpP structure and function. (a) Schematic diagram illustrating the mechanism of function of the ClpP/A–ClpP complex. ClpP/A unfolds and translocates substrate proteins into the ClpP proteolytic chamber for degradation in an ATP-dependent manner (8). ClpP/A molecules can bind ClpP from the top and bottom simultaneously. (b) ClpP side and top views. The axial pores are lined with the N-terminal residues of ClpP (13, 29) that are visible in the E. coli x-ray structure (1). (c) Detailed view of the ClpP handle region. Residues of the catalytic triad are circled. The C51 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).

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. H and 13C 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, τMIX, between 0 and 60 ms. Briefly, magnetization is transferred from 1H to 13C, and the carbon chemical shift of the starting state is recorded (t1). Subsequently, two-state longitudinal order is created that is allowed to exchange during a mixing time, followed by 13C shift evolution (t2) and transfer to 1H for detection (t3). The methyl transverse relaxation optimized spectroscopy (TROSY) principle (14) was exploited throughout the experiment. In this manner, correlations at (ωC, ωH, ωH,ωH) and (ωC,ωC, ωH,ωH) 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 \( I_{\text{cross/auto}} = A_{\text{pa}} \exp(-\tau_{\text{MIX}}) [1 - \exp(-\kappa_{\text{mix}})] + I_{\text{auto}} = B_{\text{pa}} \exp(-\tau_{\text{MIX}}) [p_a + p_b \exp(-\kappa_{\text{mix}})] \), where \( I_{\text{cross/auto}} \) is the intensity of the cross-/autopeak, \( p_a \) is the population of state a/b, \( \rho \) is the longitudinal relaxation rate of the two-state order, \( H_2C \) (where \( X \) is the Z magnetization of spin X = (H,C)), and \( \kappa_{\text{mix}} \) 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 \( \rho \) 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 \( \rho_a, \rho_b \) 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 \( \rho_a, \rho_b << \kappa_{\text{mix}} \).

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 \(-1/T\ln(1/I_0)\), where \( I_0 \) 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 13C 180° pulses are applied (20) and \( R_{\text{CPMG}} = 1/(2\delta) \), where \( \delta \) 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.

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Notably, one correlation of the pair for both I149 and I151 is narrow and intense (Fig. 2a, WT), whereas a second is significantly broader. We have assigned the narrow cross-peaks as being derived from one state (S, for slow relaxation), and the broad cross-peaks are assigned to a second state (F, for fast relaxation). Increasing the temperature leads to a broadening of the resonances of both states, indicative of an increased rate of exchange between F and S. Between 15°C and 25°C, the resonances are broadened beyond detection, and they reappear as a single, averaged peak at temperatures >35°C (Fig. 2b). Because both isoleucines show the same temperature dependence, they likely undergo a common exchange process that may reflect a concerted motion of the handle regions at the interface between the two heptameric rings. The intensities of correlations from other residues increase throughout the complete temperature range (Fig. 2c), indicating that the observed exchange process is localized.

**Kinetics and Thermodynamics of the F,S Exchange Process.** To quantify the rates and energetics of the exchange process, we measured the time-dependent transfer of longitudinal two-spin order (23, 24) between sites from a series of 3D experiments (see Materials and Methods). Fitting the decay of the autopeaks and concomitant buildup of the exchange cross-peaks enables the extraction of both exchange rates and the populations of the states F and S. The intensities of correlations from nonexchanging Ile residues increase throughout the complete temperature range (Fig. 2c), indicating that the observed exchange process is localized.

![Fig. 2. Methyl TROSY heteronuclear multiple quantum correlation spectra of U-[15N,2H], Ile [1-13C,1H] ClpP protease (300 kDa). (a) Spectrum of WT ClpP at 0.5°C. The molecular tumbling time is predicted to be >400 ns (14). The upper boxed area is enlarged in b, and the lower boxed area is enlarged in c. Spectra of the ClpP I149V and I151L mutants are consistent with both isoleucines adopting at least two distinct conformations. The dotted circles indicate the positions of cross-peaks that are eliminated by either the I149V or I151L mutation. Note the different line widths for correlations derived from the two states, indicating a difference in internal mobility for state S (slowly relaxing) and F (fast relaxing). (b) Methyl TROSY spectra of WT ClpP showing increased exchange between states F and S as a function of temperature. (c) Representative correlations from nonexchanging Ile residues.](image1)

![Fig. 3. Quantification of the F,S exchange process. (a) Longitudinal exchange spectroscopy recorded on WT ClpP at 0.5°C. The decay of autopeaks and buildup of cross-peaks is illustrated, where I/I₀ is the intensity of a given correlation normalized to the maximum intensity of the appropriate autopeak at the shortest time point used, uncorrected for any relaxation during the pulse scheme (see Materials and Methods). Experimental data points are indicated with “x,” and the dotted and solid lines correspond to the best fits of the data for states F and S, respectively (all curves are fitted simultaneously). Because of spectral overlap, the autopeaks derived from the I151 F state could not be quantified. (b) Methyl TROSY relaxation dispersion profiles recorded at 0.5°C. Contributions to relaxation rates from carbon chemical shift anisotropy as well as differences in 1H chemical shifts between states result in differences in plateau values for the curves associated with a given state (F or S) at 600 and 800 MHz. (c) Determination of the activation energies that characterize the F,S exchange process. Note that k_F → S = k_S → F so that the activation parameters are very similar for F → S and S → F. The relaxation dispersion profile of I149 recorded at 40°C is shown in Inset.](image2)
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 $k_{ex} = k_{F\rightarrow S} + k_{S\rightarrow F} = 60.8 (7.0) \text{ s}^{-1}$ and similar populations, $p_F = 0.58 (0.03)$, $p_S = 0.42 (0.03)$, 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 ($R_{\text{eff}}/\pi$) of correlations is measured as a function of the applied field strength, $R_{\text{CPMG}}$, 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 $k_{ex} = 67.5 (2.5) \text{ s}^{-1}$, $p_F = p_S = 0.5 (0.02)$ 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°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 [$\Delta H^\ddagger = 13.1 (0.6) \text{ kcal} \text{ mol}^{-1}$] and entropy [$\Delta S^\ddagger = -3.6 (2.3) \text{ cal} \text{ mol}^{-1} \text{ K}^{-1}$] 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.
under oxidizing conditions but was released from ClpP when the oxidation proceeded further than desired. The oxidized, linked form of ClpP has no proteolytic activity under reducing conditions, ClpP A153C is active in degrading the model substrate protein GFP-SsrA (26) and the fluorescent peptide Suc-LY-AMC (27), although at only ~50% of the activity of WT ClpP (Fig. 8 a and b, which is published as supporting information on the PNAS web site). This finding is consistent with our observations that mutations in the handle region often have a drastic effect on ClpP activity, most likely due to small rearrangements of the active site, which is located close to the handle region (Fig. 1a). The I151L mutant (Fig. 2a), for example, showed no proteolytic activity, although the chemical shifts of I149 and the other isoleucines were indistinguishable from WT ClpP. Stringent requirements on the orientation of the helical region might prevent activity of the unassembled or incorrectly assembled ClpP, thereby avoiding exposure of active protease sites.

The oxidized, linked form of ClpP has no proteolytic activity in peptide degradation experiments (Fig. 8a), which is most likely due to a distorted active site (see below). Surprisingly, however, when Suc-LY-AMC was added to oxidized ClpP A153C, the peptide was found to comigrate with the protease when the mixture was applied on a size-exclusion column (Fig. 8c), indicating that Suc-LY-AMC, at 200-fold molar excess over ClpP, was able to readily enter the protease lumen during the incubation period (~30 min) and was retained in the protease during size-exclusion chromatography (~45 min). In contrast, neither the reduced form of ClpP A153C nor WT ClpP showed retention of peptide (Fig. 8c).

To decouple the chemistry of substrate cleavage from peptide product release, further experiments were carried out with inactive forms of ClpP in which the catalytic Ser-111 is replaced by Ala (S111A; Fig. 4a). As with the active form of the enzyme, Suc-LY-AMC was retained in the oxidized, linked ClpP S111A A153C to a much greater extent than in the reduced, unlabeled complex (Fig. 4b Top). Similar results were obtained by using a charged FLAG peptide, DYKDDDKK (data not shown). When the ClpP fraction containing Suc-LY-AMC (Fig. 4b) was isolated from the first column (fraction 6) and passed a second time over the column, the substrate comigrated with the protease under oxidizing conditions but was released from ClpP when the protease was reduced (Fig. 4b Middle). Under oxidizing conditions, the peptide was retained in ClpP for >10 h.

This result indicates that a specific peptide-binding site is not formed within the ClpP proteolytic chamber upon oxidation of the cysteines and that the axial loops are important in mediating peptide retention. Moreover, the fact that ClpP ΔN A153C is unable to degrade substrates in the linked state but is active in the reduced form strongly suggests that the inactivity of the cysteine-linked form of ClpP is due to distortions of its active site and not its inability to release products. The rapid release of substrate from the ClpP chamber upon reduction of the disulfide is further demonstrated in Fig. 4c. Here, active ClpP has been added to a sample of S111A A153C oxidized ClpP that was first incubated with fluorescent peptide and subsequently dialyzed to remove excess peptide, and the fluorescence profile has been measured (red curve). The peptide in the S111A A153C oxidized ClpP is clearly inaccessible to the active enzyme; however, upon addition of DTT, the peptide is readily released and is cleaved by the WT ClpP, leading to an increase in fluorescence (red curve). In the control experiment, where inactive ClpP (ClpP S111A) is added to ClpP S111A A153C with retained peptide, the baseline fluorescence level is maintained throughout the time course of the experiment; no cleavage of ligand occurs (black curve).
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 molecular function.

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