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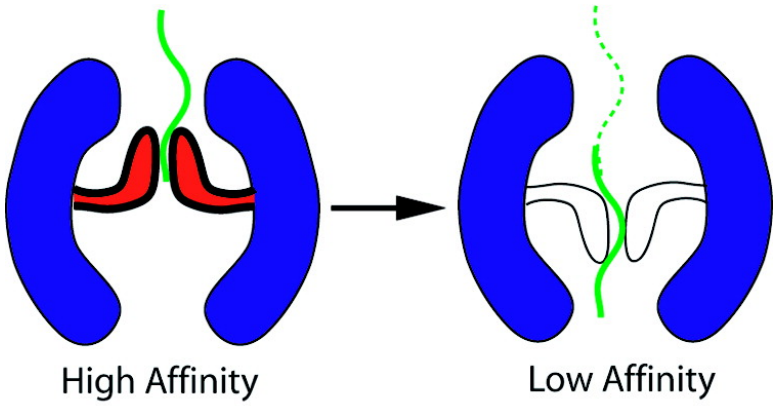
J. Am. Chem. Soc., **2007**, 129 (41), 12378-12379• DOI: 10.1021/ja074168x • Publication Date (Web): 21 September 2007



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Published on Web 09/21/2007

Single-Molecule Analysis of Nucleotide-Dependent Substrate Binding by the Protein Unfoldase ClpA

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Received June 22, 2007; E-mail: lichts@mit.edu

Many molecular machines translocate processively along polymer substrates. For motor proteins[1,2] and nucleic acid helicases,[3−6] the mechanistic basis for processive translocation appears to be nucleotide hydrolysis-dependent cycling between conformations of high and low affinity for the polymer. High-affinity binding (often associated with the ATP-bound state[2,5]) allows the machine to fasten on to the polymer without slipping backward, while low-affinity binding (often associated with the post-hydrolytic state[2,5]) allows it to move forward. Energy-dependent proteases such as ClpAP and ClpXP in bacteria or the proteasome in mammalian cells also carry out highly processive translocation along their polymer substrates.[7,8] Previous work suggests that ClpXP[9] and ClpAP,[10,11] each composed of an ATPase subunit (ClpX or ClpA) bound to a protease subunit (ClpP), employ regular switching between different conformations as part of their translocation mechanism. As in the case of motor proteins and helicases, the conformational switch observed in these energy-dependent proteases might be an ATP hydrolysis-triggered switch between discrete high-affinity and low-affinity conformations. However, the ability of Clp proteases to retain bound substrates over hundreds of ATP hydrolysis cycles[12,13] is also consistent with translocation occurring as successive "handoffs" between high-affinity bound conformations (Figure 1). To distinguish between the "alternating affinity" and "constant affinity" mechanisms, we used single-molecule fluorescence microscopy to examine the stability of ClpA/peptide complexes under conditions that mimic different nucleotide-bound intermediates in catalysis. The constant affinity mechanism predicts the observation of a single residence time for peptide substrates on ClpA, while the alternating affinity mechanism predicts the observation of at least two distinguishable residence times. The observation of a nucleotide-dependent switch between short-lived and long-lived binding modes supports the alternating affinity mechanism.

Single-molecule methods[14] are ideal for the observation of multiple conformations that might not be observable in bulk solution. We used total internal reflection fluorescence (TIRF) microscopy,[15−17] an increasingly widespread single-molecule technique, to visualize fluorescently tagged peptide substrates bound to ClpA (see Supporting Information for experimental details). A variant of ClpA containing an engineered FLAG epitope at the amino terminus was immobilized on glass coverslips coated with anti-FLAG antibodies. A peptide substrate based on the ssrA ClpA/X targeting sequence AANDENYALAA[18] was covalently tagged at its amino terminus with the fluorophore Cy3. Incubation of the Cy3−ssrA substrate with immobilized ClpA allows visualization of individual substrate molecules (Figure 2a). ClpA assembles into its hexameric, substrate-binding form only in the presence of ATP or its nonhydrolyzable analogue ATPγS,[19] and

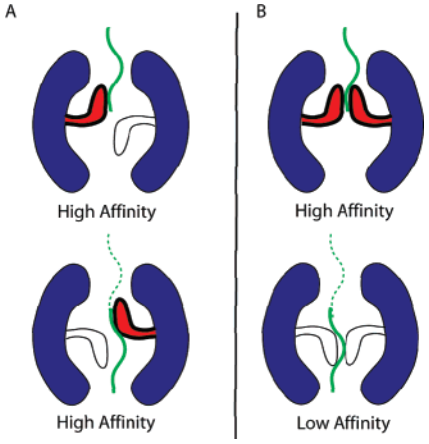

Figure 1. Possible mechanisms of substrate processing. (A) In the constant affinity mechanism, peptide substrate (in green) initially binds ClpA in a high-affinity mode (red loop). A conformational change in the peptide binding site translocates the peptide, but high-affinity binding is maintained, possibly by transfer of the peptide to a second subunit within the hexameric ring. (B) In the alternating affinity mechanism, the peptide is bound in a high-affinity conformation before translocation (upper diagram) and a low-affinity state after translocation (lower diagram).

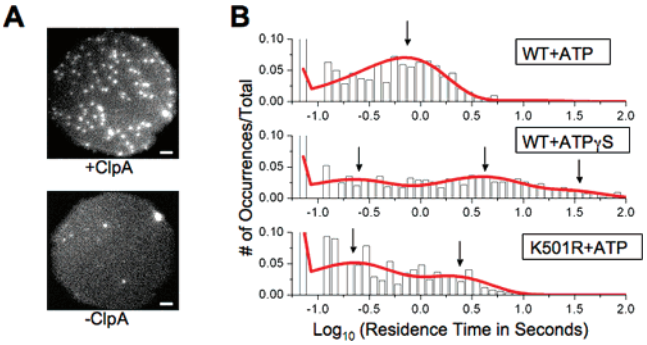

Figure 2. (A) Sample frames from experiments conducted in the presence (above) and absence (below) of ClpA. Scale bars are 2 μm. (B) Histograms indicating the number of ClpA states present in experiments conducted with wild-type ClpA and ATP (above), wild-type ClpA and ATPγS (middle), and K501R ClpA and ATP (below). Arrows indicate lifetimes of the various states.

accordingly, significant numbers (>10/frame) of substrate molecules immobilized for 100 ms or more are only observed when both ClpA and ATP or ATPγS are present.

The distribution of substrate residence times for wild-type ClpA in the presence of ATP indicates the formation of a labile bound species that is kinetically competent to be an intermediate in processive proteolysis. The ensemble turnover number for proteolytic digestion of Cy3−ssrA by ClpAP was measured to be 0.3 s^{-1} (see Supporting Information for details); assuming that translocation occurs at similar rates in the presence and absence of

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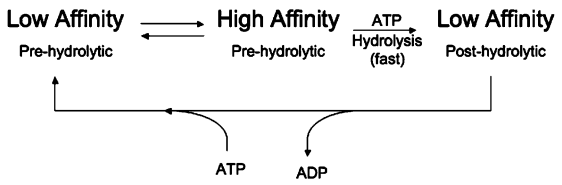
10.1021/ja074168x CCC: $37.00

Scheme 1



ClpP, one would thus expect the residence time of substrate on ClpA to be less than 3 s. The distribution of residence times was fitted using a maximum likelihood technique and has one dominant kinetic component with $\tau = 650 \pm 40$ ms. This residence time is shorter than the total residence time expected for a catalytic cycle, indicating that the observed dissociation reaction is kinetically competent for the proteolytic reaction.

The residence time for the peptide substrate was also measured under conditions where ClpA binds but cannot hydrolyze ATP. In the presence of the poorly hydrolyzable ATP analogue ATPγS,[13] ClpA can access substrate-bound conformations that are stable for much longer than observed in the presence of ATP, as well as a shorter-lived bound state (Figure 2b). The lifetimes of the three states are 3200 ± 490, 20700 ± 2800, and 170 ± 40 ms, and the relative amplitudes of the states (representing the fraction of the total events corresponding to each state) are 0.46 ± 0.04, 0.21 ± 0.04, and 0.33 ± 0.03, respectively. The analysis does not rule out the possibility that each state actually consists of multiple components; however, adding such components does not improve the fit. The long-lived states can be identified as high-affinity states; the rate at which the peptide substrate binds to ClpA is at most 2-fold slower in the presence of ATPγS compared to ATP (Supporting Information), indicating that the equilibrium constant for binding of the ATPγS-induced long-lived state to the peptide substrate is at least 2.5-fold that of the short-lived state observed in the presence of ATP. Previous biochemical work indicates that ClpA has only a single substrate binding site,[20] suggesting that the short-lived and long-lived states result from conformational rearrangement of a single binding site, rather than occupancy of high- and low-affinity sites at distinct locations in the complex.

The mutant K501R variant of ClpA, which binds ATP but exhibits severely impaired ATP hydrolysis compared to the wild-type,[21,22] was also studied. Two distinct bound states of the peptide substrate exist for this mutant in the ATP-bound form (Figure 2b): one with a lifetime of 180 ± 36 ms (amplitude 0.54 ± 0.04), and the second with a lifetime of 1700 ± 140 ms (amplitude 0.46 ± 0.04). Substitution of ATPγS for ATP does not affect these values significantly. Thus, as observed for the wild-type enzyme in the presence of ATPγS, a bound state that is stable on the time scale of seconds coexists with a much more labile bound state.

These results support the alternating affinity mechanism and are inconsistent with the constant affinity model (Scheme 1). In the alternating affinity model, ClpA in the ATP-bound state may adopt either a high-affinity or a low-affinity state, both of which are observed under conditions where nucleotide is bound but hydrolysis is impaired. Because ATP hydrolysis is rapid ($\sim 50\ s^{-1}$)[11] compared to the dissociation rate of the peptide ($< 0.3\ s^{-1}$), the pre-hydrolytic high-affinity state can efficiently be converted to a post-hydrolytic low-affinity state, explaining why the high-affinity conformation is not observed in the presence of hydrolyzable nucleotide. This mechanism is also consistent with previous studies on peptide product sizes of ClpAP, which indicate that translocation proceeds in discontinuous steps.[11] A variant of Scheme 1 in which ATP hydrolysis induces rapid equilibration of the pre-hydrolytic high- and low-affinity states might also be considered. However, because exits from the low-affinity state would dominate the kinetics for a rapidly equilibrating system, such a mechanism would predict substrate residence times similar to those of the low-affinity state, in contrast to our observations (simulations illustrating this effect are available in the Supporting Information).

The observation that ClpA can adopt a low-affinity conformation under catalytic conditions suggests that this system is able to alternate between high- and low-affinity states while maintaining high processivity. The relatively long lifetime of the low-affinity state makes such a mechanism feasible. Because even the low-affinity state is stable for an average of 650 ms, translocation will be highly processive as long as the low-affinity state is converted back to the high-affinity state with a rate constant $> 1.5\ s^{-1}$ (1/650 ms). Future work will focus on the use of longer peptide substrates to observe multiple translocation steps directly, with the goal of observing the microscopic steps in the translocation process (i.e., ATP hydrolysis, the primary translocation event, and release of the product). While much remains to be learned about the details of the translocation mechanism, the current results provide a framework for understanding how processive proteases transduce the free energy of ATP hydrolysis into the mechanical work of substrate translocation.

Acknowledgment. We thank Tania Baker and Robert Sauer for plasmids, Jeff Gelles for TIRF instrument usage, Larry Friedman for advice on experimental procedures, the David Weitz lab for Matlab scripts for particle tracking, Laura Jennings for steady-state kinetic data, Surasak Chunsrivirot for preparation of the FLAG-tagged *clpA* gene, and members of the Licht and Gershenson labs for helpful discussions.

Supporting Information Available: Supplementary methods and experimental procedures, movies of TIRF experiments in presence of ATP and ATPγS. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA074168X