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Mu Transpososome Architecture Ensures that Unfolding by ClpX or Proteolysis by ClpXP Remodels but Does Not Destroy the Complex

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Summary

The Clp/Hsp100 ATPases are protein unfoldases that both alter protein conformation and target proteins for degradation. An unresolved question has been how such seemingly destructive enzymes can "remodel" some protein substrates rather than destroy them. Here, we investigate the products of ClpX-mediated remodeling of a hyper-stable protein-DNA complex, the Mu transpososome. We find that although an oligomeric complex is maintained, release of some subunits accompanies ClpX action. Replacement of transposase's endogenous ClpX-recognition sequence with an exogenous signal reveals that the mechanism of remodeling is independent of both the recognition signal and the identity of the unfoldase. Finally, examination of the transposase-DNA contacts reveals only a localized region that is altered during remodeling. These results provide a framework for protein remodeling, wherein the physical attributes of a complex can limit the unfolding activity of its remodeler.

Introduction

Many multistep biological pathways are controlled by progression through a series of distinct multiprotein complexes. To transition from one complex to the next, either the first complex is destroyed and a new one replaces it, or the complex is altered to fit a new role. We refer to this second alteration as "protein remodeling," a process in which an enzyme promotes a conformational change in a target protein to generate a biologically active product. A common example of protein remodeling is the destabilization of multimeric complexes. The Clp/Hsp100 ATP-dependent unfolding enzymes, originally identified for their role in protein degradation, are also remodelers of protein complexes. Thus, a key question has been how such seemingly destructive enzymes remodel some protein substrates rather than destroy them. The recombination complex made by the bacteriophage Mu (called a transpososome) is particularly well suited to study the mechanisms and products of such a remodeling event [1, 2]. The biochemical steps of transposition are well characterized [3–5], and numerous advances have been made in understanding the function and mechanism of the Mu transpososome's remodeler, the *E. coli* ClpX protein.

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ClpX, a member of the Clp/Hsp100 ATPase family, leads a dual life as both solitary chaperone and regulatory subunit of the two-compartment ClpXP protease [6, 7]. The molecular mechanism of the ClpXP (and related ClpAP) degradation pathway has been elucidated in detail. First, ClpX selectively recognizes specific peptide signals exposed on a target protein; one well-characterized recognition signal is the ssrA tag. SsrA is an 11 amino acid peptide sequence that is added cotranslationally to growing polypeptide chains when translation stalls prematurely [8, 9]. After binding the recognition sequence, ClpX unfolds and directionally translocates the protein into the ClpP peptidase compartment of the protease [10–15]. The active sites in ClpP cleave the target protein into short peptides [16–18]. The highly processive nature of the unfolding reaction ensures complete degradation of the target protein [19].

As a remodeling enzyme, ClpX appears to use the same protein unfolding mechanism as for degradation, but for a milder biological outcome [20]. Both ClpX and ClpA have a cognate peptidase, ClpP, and are therefore likely to be present in the cell as ClpXP and ClpAP. ClpX and ClpA have numerous substrates, and in many cases it is yet unknown whether remodeling or degradation may be the biologically relevant action on the target protein. In the case of the Mu transpososome, however, genetic analysis has established that the action of ClpX is essential but that the absence of ClpP has little effect [21]. A key question has been whether it is truly necessary for ClpX to function alone to remodel the transpososome or whether ClpX and ClpXP could in fact produce the same outcome.

Mu uses transposition to replicate its genome during lytic development. The 75 kDa transposase (MuA) brings together the left and rights ends of the Mu genome by binding to specific end sequences to form the transpososome [22–24]. The long asymmetric DNA sequences (R1, R2, R3, L1, L2, and L3) are named based on their proximity to the terminal base pairs on the right and left ends of the Mu genome. Once assembled, the transposase subunits in the transpososome catalyze the DNA cleavage and joining reactions necessary for recombination. The transpososome changes at each major step in the recombination pathway, becoming increasingly thermodynamically stable [25]. Completion of DNA joining produces the most stable transpososome, termed a strand-transfer complex (STC). The stable core of the STC is made up of four MuA subunits, and this core exhibits remarkable stability, although the protein-protein and protein-DNA contacts are all noncovalent [24, 26]. The STC resists extreme heat and high salt in vitro, and the presence of the STC inhibits replication of the Mu genome [21, 25, 27]. The STC is, however, vulnerable to the activity of ClpX [28]. ClpX recognizes a specific peptide sequence at the extreme carboxyl terminus of the transposase and uses its unfolding activity to remodel the STC, resulting in an altered complex known as the STC2 [29].

The STC2, in contrast to the STC, is a very fragile

complex. It falls apart during native gel electrophoresis and under high salt challenge [20, 28, 29]. There is limited structural information about the STC2; it was demonstrated to have oligomeric properties and maintain the Mu DNA ends in a synapsed state [29]. Further, the STC2 plays a critical role in the Mu life cycle. It is required for proper recognition of the recombination sites by as yet unidentified cellular factors, termed Mu replication factors; these factors, in turn, appear to be needed to assemble replication forks at the Mu recombination sites [30].

Previous studies suggested that the ability of ClpX to promote global unfolding may be responsible for remodeling [20]. An unresolved question, however, has been whether protein remodeling can occur with or without associated protein degradation. Here, we provide new evidence suggesting that limited protein degradation can accompany successful remodeling by demonstrating that another unfoldase of the Clp/Hsp100 family, coupled with a peptidase subunit, can generate the same remodeled product as ClpX. Consistent with this idea, we show that transpososome remodeling by the unfoldase alone is accompanied by release of some constituent subunits. Furthermore, we demonstrate that the decision to remodel rather than destroy the complex is not determined by the ClpX recognition sequence or by the identity of the unfoldase, suggesting instead that the outcome may be determined by the physical attributes of the transpososome. Finally, by analyzing protein-DNA contacts present in the transpososomes, we demonstrate that remodeled complexes have a preferred configuration, with contacts distinct from those prior to remodeling. These data provide new insight into how either unfolding by ClpX or proteolysis by ClpXP can remodel but not destroy the Mu complex, and why initiation of Mu-specific DNA replication is targeted to the left end of the genome.

Results

ClpX and ClpA Both Destabilize MuA-ssrA Transpososomes

Previous experiments suggested that the unfolding activity of ClpX could be responsible for transpososome remodeling [20]. A prediction of this hypothesis is that a second, quite unrelated unfolding enzyme could substitute for ClpX provided that it could recognize the transposase. To test this hypothesis, we constructed a fusion protein in which the carboxy-terminal domain of MuA was replaced with the ssrA sequence to make MuA-ssrA. The C-terminal domain of MuA, which contains the endogenous ClpX-recognition sequence, is not required for assembly or stability of complexes or for recombination activity [31]. SsrA is efficiently recognized by both ClpX and ClpA in vitro [32]. ClpA, also an unfolding enzyme in the Clp/Hsp100 family, is nonetheless substantially different than ClpX. ClpA is almost twice the size of ClpX and has two ATPase domains, whereas ClpX only has one ATPase domain. Furthermore, ClpX and ClpA generally recognize different proteins, although both recognize ssrA-tagged substrates. Thus, fusing the ssrA tag to MuA allowed us to ask two questions. First, does ClpX remodel transpososomes containing MuA and MuA-ssrA in the same manner? And if so, can a distinct unfolding enzyme also remodel the transpososome?

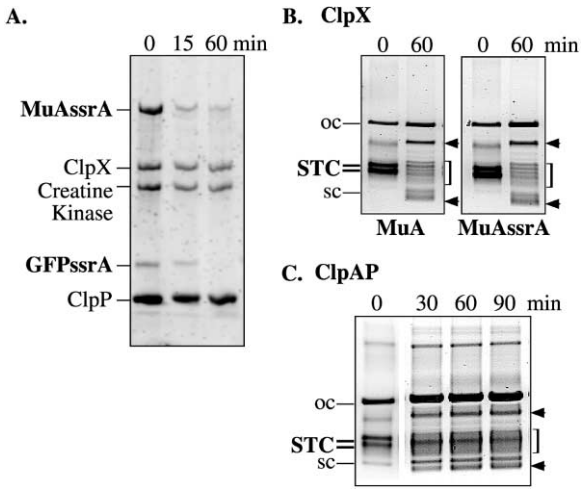

Figure 1. ClpX and ClpA Can Destabilize MuA-ssrA Transpososomes

(A) Time course of degradation of MuA-ssrA monomers by ClpXP. Creatine kinase is present as part of an ATP-regeneration system. GFP-ssrA degradation was used as an internal control.

(B) ClpX destabilization of MuA and MuA-ssrA strand transfer complexes (STCs). Arrows and brackets designate released strand transfer products (rearranged DNA molecules) that are released and appear as new bands upon destabilization of the STCs [52, 53]. Mini-Mu plasmids were allowed to undergo intramolecular strand transfer in the presence of MuA. Unreacted plasmid DNA is denoted as "sc" and "oc" (supercoiled and open circular). DNA was visualized by Vistra Green staining.

(C) ClpAP destabilization of MuA-ssrA STCs on mini-Mu plasmids as described for (B).

To verify that monomers of the MuA-ssrA were bona fide ClpX substrates, we checked for degradation by ClpXP in vitro (Figure 1A). As expected, ClpXP degraded the protein in an ATP-dependent fashion (Figure 1A and data not shown). MuA-ssrA and GFP-ssrA, our standard model substrate, were degraded with similar kinetics.

To address whether ClpX recognizes and destabilizes the MuA-ssrA transpososomes, we determined whether, after ClpX treatment, the complexes were stable during native gel electrophoresis. As previously demonstrated, the transpososomes that have undergone strand transfer to form the strand-transfer complex (STC) remain stable during native gel electrophoresis, whereas ClpX-remodeled complexes (STC2) fall apart and thereby release the recombined DNA products [20, 29]. Indeed, ClpX destabilized both wild-type STCs and MuA-ssrA STCs (Figure 1B).

Next, we asked whether ClpA could also destabilize transpososomes containing MuA-ssrA. ClpA alone did not render the complexes unstable to gel electrophoresis; however, ClpAP did destabilize the MuA-ssrA complexes (Figure 1C). This reaction was also ATP dependent (data not shown). These experiments demonstrate that both ClpX and ClpA can destabilize transpososomes, likely using their common ability to promote ATP-dependent protein unfolding. We expect that the ClpP requirement in the ClpA-mediated remodeling re-

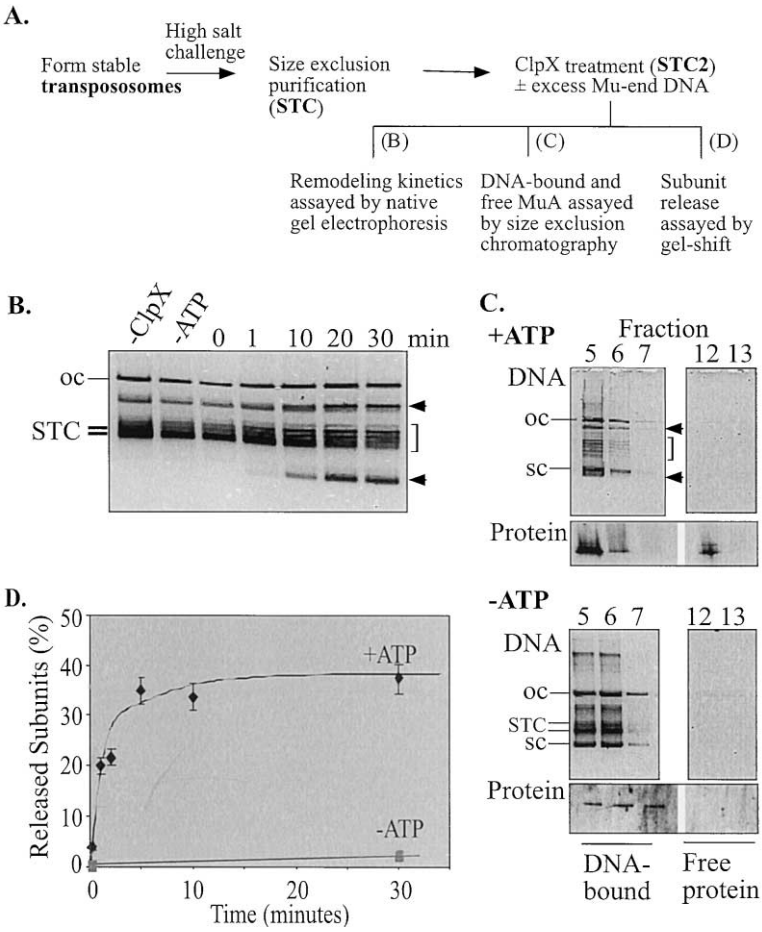

Figure 2. Remodeling Is Accompanied by Release of Transposase Subunits

(A) Scheme of the methods used to analyze the products of ClpX-mediated remodeling.

(B) Time course of transpososome destabilization.

(C) Gel filtration fractions of ClpX-treated complexes (30 min time point, ±ATP). The upper panel is a native agarose gel strained with Vistra Green to view DNA, and the lower panel is a Western blot probed with anti-MuA antibody. Plasmid and plasmid-bound transposase elute in fractions 5–7, and unbound transposase elutes in fractions 12 and 13. Fluorimager files for the Western blot were printed using the linear grayscale with the black (100%) setting to allow for visualization of the transposase in fractions 12 and 13. Therefore, the transposase band in fraction 5 is roughly 3-fold overexposed. Quantitation gives a lower limit of 17% for transposase in fractions 12 and 13.

(D) Quantitation of the time course of subunit release in the presence of ClpX and ATP (diamonds). Released subunits were detected by their ability to shift the mobility of DNA fragments containing strong transposase binding sites. Data plotted are averaged from three experiments with standard errors shown. The DNA fragments did not cause release of subunits from the STCs in the absence of ClpX (data not shown) or ATP (squares).

actions may be due to the stabilizing affect of allosteric interactions between ClpA and ClpP. ClpP itself does not contribute to the unfolding activity of the complex, and numerous experiments have demonstrated a stabilizing effect of ClpP for both ClpX and ClpA [31, 33]. Therefore, we conclude that the intrinsic unfolding activity of a Clp/Hsp100 enzyme is sufficient to destabilize transpososomes. In the following sections, we address the structural consequences of transpososome remodeling by ClpX, ClpXP, and ClpAP using wild-type transposase and MuA-ssrA.

Release of Transposase Accompanies Remodeling

The ability of these two distinct unfoldases, ClpX and ClpAP, to destabilize the strand-transfer complex provided further evidence that protein unfolding is indeed the mechanism underlying remodeling. Current models posit that ClpX and ClpA unfold their degradation substrates in a processive manner such that the ATPase moves along the polypeptide chain from the recognition signal [11]. When this same mechanism is applied to remodeling in the absence of a peptidase component, we predict that processive unfolding would cause the ClpX-contacted transposase subunit to be released from the complex. To look for subunit release, we returned to the native system using wild-type MuA and ClpX without ClpP and developed a quantitative gel-shift assay. This method allowed us to detect the presence of any free transposase that may be released by ClpX action by the ability of these subunits to bind to DNA fragments with strong transposase binding sites. Earlier work demonstrated that transposase unfolded by ClpX efficiently regains DNA binding activity [20].

We assembled transpososomes, purified stable STCs, subjected them to ClpX, and analyzed the reaction products (Figure 2A). As expected from previous observations, remodeling was nearly complete within the first 10 min of incubation with ClpX, as assayed by native gel electrophoresis (Figure 2B). Furthermore, after this incubation period most of the transposase was still bound to the DNA, as measured by its coelution with the DNA during gel filtration (Figure 2C). However, we also clearly detected the appearance of free transposase during the first 10 min of the ClpX treatment. Quantitation of the DNA binding assay revealed that about 35% of the transposase subunits were released from the complexes in a ClpX- and ATP-dependent manner (Figure 2D). Likewise, some free transposase was detected in the later eluting fractions in the gel filtration experiment (Figure 2C, fractions 12 and 13).

Importantly, in the absence of ATP no detectable transposase eluted in the later fractions, and the complexes remained stable during gel electrophoresis (Figure 2C, lower panel). This confirms that the STCs did

not spontaneously fall apart during treatment, but rather that both destabilization and subunit release were active processes dependent upon the ClpX ATPase. Another potential explanation for the observed subunit release is that ClpX could remodel the complexes, to make them fragile, and a small fraction of these complexes might then fall apart to generate the released subunits. However, if this were occurring, naked DNA products should also be released; we would expect this DNA to be visible by gel electrophoresis following crosslinking of the STC2 complex, but none was observed (data not shown, see footprinting section under Experimental Procedures). Finally, the time course in Figure 2D demonstrates that no additional subunit release occurs after the initial incubation with ClpX and ATP, suggesting that release is not a result of spontaneous disassembly of the STC2 but rather a direct consequence of ClpX action.

Products of Remodeling Are Independent of the Tag and the Identity of the Unfoldase

To investigate what controls protein unfolding to cause complex destabilization and limited subunit release rather than total destruction, we used transpososomes constructed with MuA-ssrA. One possible controlling feature is the endogenous ClpX-recognition sequence on MuA that might provide a remodeling-specific signal in the context of the transpososome. The ssrA tag normally targets proteins for complete unfolding by ClpX and degradation by ClpXP. Therefore, we analyzed the products of ClpX-catalyzed destabilization of MuA-ssrA STCs to determine whether fragile transpososomes were formed or whether the transpososomes were completely destroyed. As shown earlier, ClpX clearly destabilized the MuA-ssrA complexes (Figure 3A). Importantly, immunoblots of the size exclusion chromatography fractions revealed the same distribution of MuA-ssrA as for complexes consisting of wild-type transposase (Figure 3A, lower panel; compare to Figure 2C); most of the transposase originally in stable complexes remained associated with the recombined DNA. Measurement of released transposase by the gel-shift assay confirmed that despite the exogenous recognition tag, remodeling of the complexes resulted in release of only a fraction of the constituent subunits from the complex (Figure 3B). These results suggest that the endogenous ClpX-recognition sequence on the Mu transposase does not provide remodeling-specific information to ClpX. Likewise, we conclude that the presence of a sequence that normally targets proteins for degradation is not sufficient to trigger complex destruction by ClpX.

Similarly, we asked whether the identity of the unfoldase controls the outcome of the protein-processing reaction. Therefore, we determined whether products of remodeling mediated by ClpAP also include only limited subunit release and a destabilized complex on the DNA. Indeed, although ClpAP destabilized the complexes, size exclusion chromatography revealed that most of the transposase remained associated with the recombined DNA (Figure 4). The requirement for ClpP in this case prevented a measurement of released subunits, which were degraded. However, the combination of the

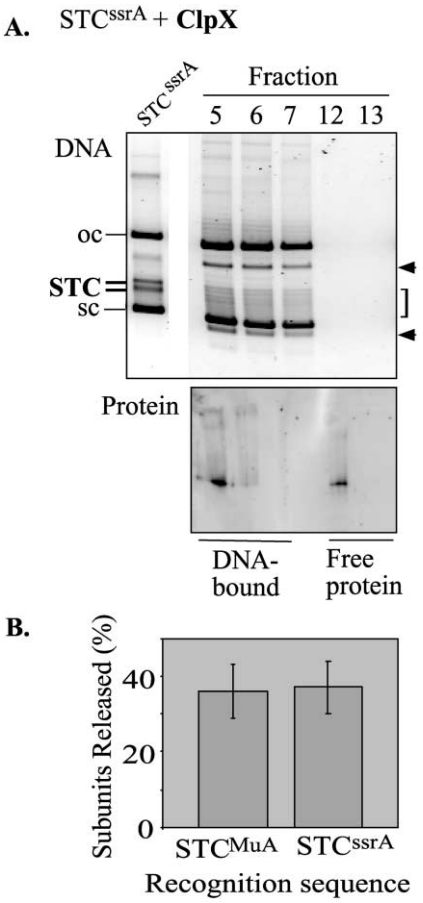

Figure 3. Remodeling Is Independent of the ClpX-Recognition Tag

(A) Gel filtration fractions after ClpX remodeling of MuA-ssrA complexes. Destabilized strand transfer products are shown in the upper panel. Arrows and bracket designate released DNA products. In the lower panel, fractions 5–7 contain the DNA-bound transposase, and fractions 12 and 13 contain the free protein. Fluorimager files for the Western blot were printed using the linear grayscale with the black (100%) setting to allow for visualization of transposase in fractions 12 and 13. Therefore, the transposase band in fraction 5 is roughly 2.5-fold overexposed. Quantitation gives a lower limit of 20% for transposase in fractions 12 and 13.

(B) Comparison of the total percent of subunits released (determined by gel shift) from MuA and MuA-ssrA complexes after a 30 min incubation with ClpX.

presence of MuA-ssrA with the recombined DNA and the destabilized nature of the complexes strongly suggest that the structural outcome of remodeling was very similar, whether the unfoldase was ClpX or ClpA. Furthermore, we observe the same gel filtration profiles after remodeling of MuA-ssrA with ClpXP (data not shown). These data demonstrate for the first time that the outcome of remodeling is the same for the complex regardless of the presence or absence of the peptidase component of the protease. The only difference for the outcome is in the fate of the subunits contacted by the unfolding enzyme. Thus, we conclude that neither the specific tag, the identity of the unfoldase, nor the presence of an associated peptidase determines the structure of the remodeled complex.

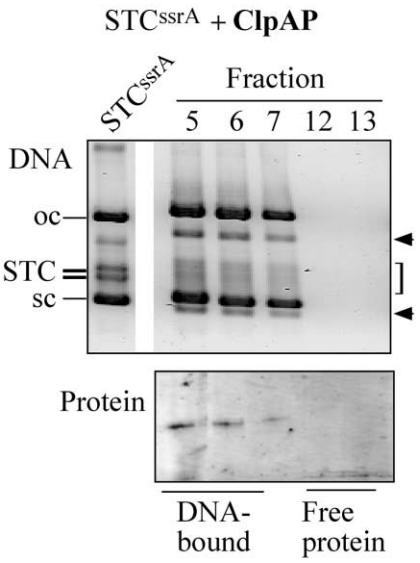

Figure 4. Remodeling Is Independent of the Identity of the Chaperone

Gel filtration fractions of ClpAP-treated MuA-ssrA complexes. DNA and associated protein elute in fractions 5–7, and free transposase elutes in fractions 12 and 13. The lack of detectable free transposase by immunoblot in fractions 12 and 13 is due to degradation of those subunits by ClpAP.

Remodeling Alters Specific Transposase-DNA Contacts

The experiments presented above indicate that remodeling of the transpososome is not directed by the recognition sequence or by the unfoldase. Furthermore, we observe release of subunits from the complex as a result of remodeling. Therefore, we asked whether the STC2 might reach a terminal state with a unique or at least preferential subunit configuration. This is the outcome expected if ClpX contacts, unfolds, and removes a specific subunit of the STC to generate the STC2 that is then refractory to further unfolding by ClpX. To investigate this, we looked at changes in the DNase protection pattern of the Mu end sequences when comparing the STC and the STC2. The STC produces a three-site footprint, protecting the R1, R2, and L1 Mu end sequences from DNase cleavage (Figure 5A). In the presence of competitor DNA, the transpososome does not interact strongly enough with the L2 sequence to produce a clear footprint [34] (see also [24]). Thus, this experiment probed for differences in DNA binding by transposase around three end sites, L1, R1, and R2.

Transpososomes were prepared and then the samples were split into ClpX-treated and mock-treated reactions (see Experimental Procedures). We verified that remodeling went to completion in the ClpX-treated reactions by checking a portion of the sample in the gel destabilization assay. The samples were crosslinked with gluteraldehyde to maintain their integrity through the rest of the procedure; the crosslinked STC2s had the same mobility as the STCs in the gel electrophoresis assay (data not shown). After treatment with DNase, the STCs and STC2s were isolated from unreacted DNA on a native gel; similar amounts of DNA were recovered for the two types of complexes. The DNase pattern confirmed that the L1 region (bp 1–30) was protected in the STC (Figure 5B). Strikingly, upon treatment with ClpX, DNA contacts were altered in both the L1 end site and the neighboring ~60 bp region within the Mu genome. Some changes suggest deprotection, whereas others appeared to be newly created hypersensitive sites. No obvious changes were observed in the DNA outside the cleavage site of the left end (flanking DNA). Furthermore, analysis of footprints at the right end revealed no changes in protection of the R1 and R2 binding sites or in the flanking DNA (Figure 5C). The specific changes in the L1 region were also observed when the order of crosslinking and DNase treatment was reversed (data not shown). These data demonstrate that ClpX remodeling of transpososomes preferentially disrupts protein-DNA contacts at the left end of the Mu DNA sequence.

Discussion

The Clp/Hsp100 ATPases are best known for their involvement in proteolysis. Most of the mechanistic studies of these proteins have been done in the context of degradation. Processive unfolding and translocation into the proteolytic chamber leads to peptide fragments 9–20 amino acids in size [11–14, 19]. In contrast, although it is clear that products of Clp/Hsp100-mediated remodeling are important biologically, the mechanism and structural consequences of remodeling have been unclear. Here, we have analyzed the outcome of ClpX-mediated transpososome remodeling to further understand the link between the destructive unfolding activities of ClpX and the biologically active complex it leaves behind after remodeling. Our data provide new evidence suggesting that structural attributes of the original stable complex can direct remodeling by either the unfoldase alone or the unfoldase coupled with a peptidase. Furthermore, we show that the cues provided by the complex are so robust that neither the recognition sequence nor the identity of the unfoldase alters the products. Finally, we demonstrate that the remodeled complex has a preferential configuration that may explain the asymmetric replication initiation at the Mu DNA ends.

Previous experiments clearly demonstrated that an oligomer of transposase remains associated with the recombined DNA. Our experiments now reveal that there is a second product of remodeling: free transposase monomers. The presence of this released transposase is the result predicted by models in which ClpX functions by processive protein unfolding; therefore, these data further support the hypothesis that ClpX remodeling depends on its unfoldase activity. Since only a minority of the subunits in the transpososome are released and therefore are likely unfolded by ClpX, these results reconcile how protein unfolding can cause destabilization rather than destruction of the complex. These data are in agreement with a published study demonstrating the continued presence of transposase at the recombination sites after remodeling [29].

Our detection of free transposase released during re-

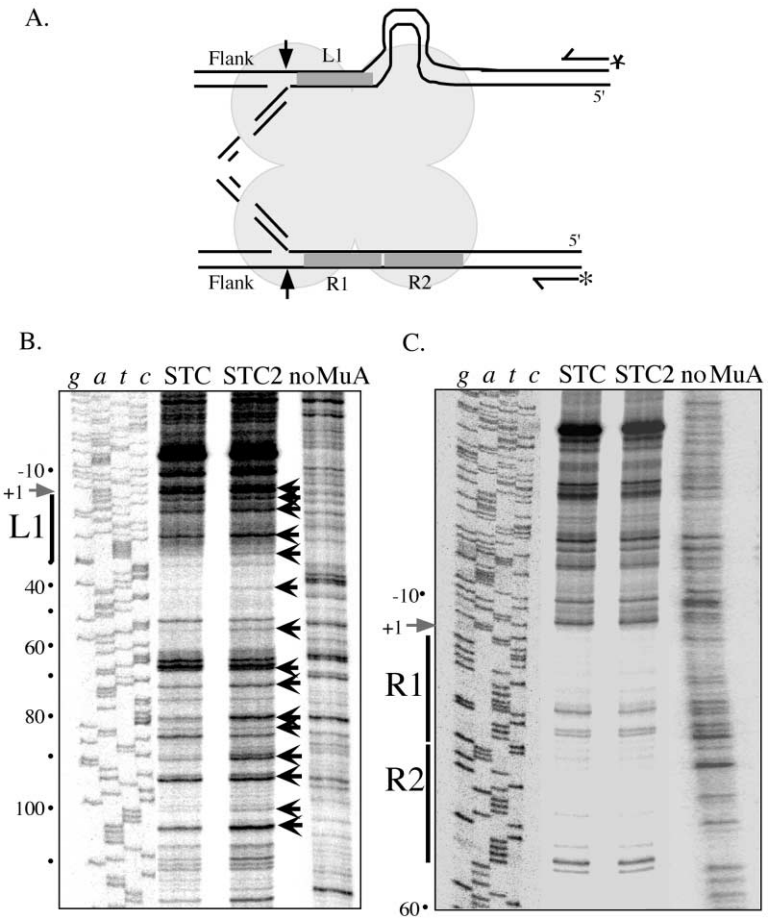

Figure 5. DNase Protection Pattern of the Mu Genome Ends in the STC2

(A) Schematic of the left and right ends of the Mu genome. Light gray lobed shape denotes transposase subunits. Dark gray boxes denote Mu end binding sites analyzed in this experiment. Regions of DNA protected from DNase in the STC include these sites and almost 20 nt outside the cleavage site. Arrows indicate the cleavage site (+1 bp) on each end of the DNA.

(B) DNase protection patterns for the Mu genome left end. Complexes were assembled and treated with ClpX as described for the preceding experiments. To ensure that subunits removed by ClpX did not rebind the Mu end sequences in the complexes, a competitor DNA containing two strong MuA binding sites was added. (High salt could not be used, as this treatment would dissociate all subunits from the ClpX-destabilized complexes.) After ClpX treatment, complexes were crosslinked with gluteraldehyde. This step, although not necessary for the stable STC, was necessary to maintain the STC2 during subsequent steps that included higher salt concentrations. The plasmid DNA was digested with restriction enzymes and with DNase I, as previously described [24, 34]. The crosslinked complexes with synapsed Mu ends were purified by nondenaturing polyacrylamide gel electrophoresis, and the DNase-digested sites in the recovered DNA were detected by primer extension. The L1 binding region (bp 4–30) is indicated left of the ladder. The cleavage site is designated with an arrow at the left. Newly accessible DNA in the ClpX-treated samples, including appearance of new bands and increasing intensity of others, is denoted with arrows at right. DNase-treated mini-Mu DNA with no MuA is at right. Plasmid DNA was incubated with all proteins except MuA and treated with DNase in the presence of competitor DNA fragments. Relative recovery after DNase treatment was slightly lower than for DNA in complexes.

(C) DNase protection pattern of the Mu genome right end. The R1 and R2 binding sites are indicated left of the ladder. The cleavage site is designated with an arrow.

modeling by ClpX presents an interesting challenge to the current thinking about the oligomeric state of the STC and the STC2. Early protein-protein crosslinking experiments demonstrated that STCs contain a tetramer of MuA [24, 29, 35, 36]. Other experiments showed that additional more loosely associated subunits could be present and activate the complex [37]. The first crosslinking experiments examining the oligomeric properties of the STC2 suggested that the STC2 is also a tetramer [29]. However, those data clearly show an abundance of trimers, dimers, monomers, and even higher-order species. Crosslinking results depend heavily on the solution conditions, and the most informative crosslinking conditions for the core tetramer are incompatible with the conditions of ClpX remodeling. Crosslinking studies of the STC and STC2, in our hands, produce similar results to those published, but these conditions are clearly not optimized to capture a single stable species [34]. Thus, our data are in agreement regarding the continued presence of an oligomer but not necessarily a tetramer at the recombination site. While we favor the idea that ClpX removes a core subunit during remodeling, ClpX action on a peripheral subunit may also occur. In either case, the removed subunit is one that is probably not efficiently crosslinked under the reaction conditions used for remodeling. Clearly, understanding the architecture of the different forms of the transpososome is becoming increasingly important to understanding the recombination pathway as a whole. Experiments using sensitive biochemical and structural probes to study these complexes are underway.

Unfolding Activity, Not the Recognition Sequence or the Specific Unfoldase, Is Responsible for Remodeling

The ability of Clp/Hsp100 proteins to produce biologically important complexes via remodeling presented the possibility that these enzymes might use specific mechanisms to prevent degradation of some protein substrates. In the case of transpososomes, the ClpX-recognition signal on the transposase is one obvious candidate for defining a remodeling-specific interaction. By replacing the endogenous ClpX-recognition sequence with an exogenous tag, we found that the sequence of the recognition tag does not switch the activity of ClpX from remodeling to destruction. The other obvious possible determinant

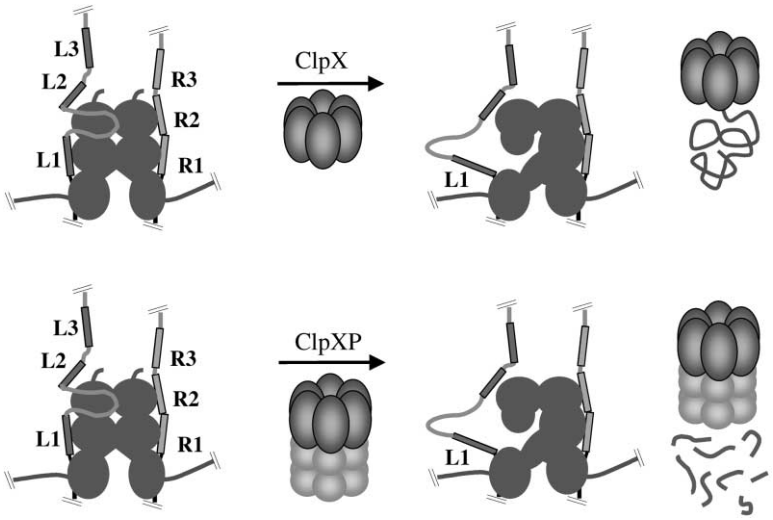

Figure 6. Model for Transpososome Remodeling

Two products result from ClpX-mediated remodeling: the destabilized complex and the released subunit(s). The key features of the model hold whether the active remodeling enzyme is ClpX alone or ClpXP. First, ClpX uses its unfolding activity when it interacts with transposase subunits in the strand-transfer complex. Second, only one subunit or a small subset of subunits is processively unfolded, and therefore only a subset is released from the complex into solution (or degraded). Finally, the remodeled complex has a preferred configuration in which changes in DNA accessibility in and around the L1 binding region are observed. This region of newly accessible DNA might serve as loading site for the host replication machinery.

for remodeling was the identity of the unfoldase. Of the four Clp/Hsp100 ATPases in *E. coli*, ClpX is uniquely essential for transpososome remodeling in vivo [21]. However, we have found that the identity of the unfoldase used for remodeling is interchangeable, provided an appropriate substrate recognition signal is present. Thus, our data not only confirm that the unfolding activity of the Clp/Hsp100 chaperones is sufficient to explain remodeling, but they also put to rest the notion that the endogenous ClpX-recognition signal produces a special interaction between MuA and ClpX. Neither the tag nor the enzyme conveys any remodeling-specific information to the outcome of the reaction. Instead, our results support the idea that some characteristic of the stable STC limits unfolding and degradation to a single subunit (see below).

Structure Determines Remodeled Structure

Comparison of the specific DNA contacts made by the transpososome before and after remodeling reveals that the DNA near one Mu end site (L1) becomes preferentially accessible to DNase after remodeling. In contrast, we do not observe changes in the R1, R2, right flank, or left flank contacts. These data indicate that ClpX interaction with the complex involves rearrangement or even release of the L1-associated DNA contacts. Transposase subunits bound to the left end of the Mu DNA may be preferential targets for ClpX, as there is already only weak association of the transposase to the L2 site, and the DNA is severely bent between L1 and L2. Since we observe changes in the L1 region, perhaps the simplest model for this change in protection state is a direct interaction between ClpX and the L1-associated subunit. However, as the L2 site is not protected in either the STC or STC2 under the conditions studied, it is also possible that the L2-associated subunit is directly contacted and unfolded by ClpX. Since the L2-associated subunit binds the DNA weakly, this feature could make it a unique target for ClpX. Thus, either direct unfolding of the L1- or L2-bound subunits could give rise to the observed changes in DNA protection.

Our previous work demonstrated that ClpX could contact and unfold a limited number of subunits in the transpososome to destabilize the complex. We now understand that there are two products of ClpX-catalyzed remodeling: the destabilized protein-DNA complexes and the released subunits. These findings, combined with the previous results, allow us to present a more detailed model of the mechanism and products of the Mu transpososome remodeling reaction (Figure 6). In this model, ClpX specifically contacts a limited number of subunits and unfolds them. The unfolding action removes key protein-DNA contacts in and around the L1 DNA site, rendering accessible a large region of previously protected DNA. Whether one or perhaps sometimes two subunits are released from the STC, the remaining subunits must be sufficient to maintain the Mu ends in a synapsed form (see Experimental Procedures; [29]). The continued protection of the left flanking DNA (presumably by the R1-bound subunit) and the remaining left-end subunit may provide the contacts on the left end necessary to maintain synapsis. An important element of this model is that the remodeled complex is the same whether ClpX or ClpXP is the active remodeling enzyme, although the released subunit in the latter case will likely be degraded. The ability of either enzyme to perform remodeling may be critical to the transition in the cell, since no obvious regulation exists to prohibit ClpXP from interacting with the transpososome. Thus, specific physical cues that guide the remodeling reaction must reside in the stable complex itself. For example, only one of the ClpX-recognition signals might be available for interaction and unfolding by ClpX. As discussed above, the transposase binding sites are arranged in an asymmetric fashion at the ends of the Mu genome. Therefore, we speculate that this could help to define the structural asymmetry in the STC that may direct the remodeling reaction.

Previous work from the Nakai lab demonstrated that the STC2 is specifically recognized by additional factors that aid in replication fork assembly [27, 29]. Thus, we suggest that the newly exposed DNA and transposase surfaces at the left side of the transpososome could unmask the interaction site for these factors. Klenow

fragment extension assays demonstrated that the 3′ hydroxyls remain protected after ClpX-mediated remodeling [33]. This is consistent with our observation that the DNA at and flanking the cleavage site on either end is not altered by remodeling. Protection of the flanking DNA at the left end could be provided by the catalytic domain of the R1-bound subunit that cleaves the left end of the Mu DNA [38]. Furthermore, comparison of the Mu core domain structure with the cocrystal structure of Tn5 transposase and DNA suggests that domain IIB of the L2-bound Mu transposase subunit may bind the left-end flanking DNA [3, 39]. The continued presence of the remodeled complex at the Mu recombination site could serve to protect the DNA ends until the presence of replication machinery is assured.

Along the transposition pathway, the transpososome undergoes a series of changes such that each version of the complex is uniquely suited to its function and temporal position in the pathway. The left and right Mu DNA ends are clearly different, and this asymmetry undoubtedly plays important roles in directing transitions along the recombination pathway. The L1 site was recently demonstrated to function as a "trigger" in which formation of protein-DNA contacts at L1 provides the final commitment step in forming a stable complex [40]. The data presented here suggest that once recombination is complete, ClpX-mediated release of the L1-associated contacts may again act as a trigger during remodeling. This conversion is not merely a reversion to the pre-STC state (known as the LER) but rather is a forward progression to the STC2, which has distinct protein-DNA contacts from the STC and the LER. The specific deprotection of the extreme left end of the Mu genome could facilitate replication fork assembly and therefore may explain the observation that replication proceeds preferentially from the left end of the Mu genome [10, 41, 42].

Significance

AAA$^+$ enzymes are widespread and have a broad range of functions. Some family members, including the Clp/Hsp100 enzymes, function both in protein degradation and protein remodeling. These enzymes appear to use the same protein unfolding mechanism to achieve these disparate goals. How do these enzymes produce such distinct biological outcomes? We addressed this question by analyzing the products of Mu transpososome remodeling by ClpX, ClpXP, and ClpAP. We found that the contacted subunits were unfolded and released or unfolded and degraded, depending on the enzyme that carried out the reaction. In contrast, the remodeled complex had the same structure, regardless of (1) the recognition sequence used to recruit an unfoldase, (2) the identity of the unfolding enzyme, or (3) the presence or absence of a peptidase associated with the unfoldase. Our findings suggest that remodeling occurs when the protein unfolding and/or degradation activity of a AAA$^+$ enzyme is restricted by the structure of the substrate.

These mechanistic insights help guide our thinking about the molecular events accompanying other remodeling reactions. For example, the AAA enzyme NSF, which promotes membrane fusion, remodels and destabilizes SNARE complexes [43–45]. We propose that NSF could selectively unfold individual SNARE components, thereby releasing intact subunits capable of reassembling for further rounds of fusion. The proteasomal 19S regulatory complex is another putative remodeling enzyme made of AAA$^+$ subunits. This 19S component, in the absence of the 20S protease, stimulates transcription by RNA polymerase II [46, 47]. Perhaps selective unfolding and removal of a constituent subunit of the transcription complex could remodel these complexes to activate Pol II. Since the 19S complex regulates degradation by the proteasome, much like ClpX does for ClpP, it is intriguing to consider that the ATPases of the 19S complex could use the same mechanism to promote both remodeling and degradation.

Experimental Procedures

Materials

The ssrA-coding sequence was cloned into pWZ170 at unique EagI and BamHI sites using primers TB258 and TB773 (3′-GACGACGGC GTCTATCGGCTTGGTCGTCGTGGTCTTAGTCAGCGTTTGCTGCTT TTGATGCGAAATCGTCGAATTCCTAGGAAAA-5′) [48]. MuA and MuA-ssrA were purified as described for MuA [35, 38]. ClpX, ClpA, and ClpP were purified as described [12, 18, 49]. All ClpX and ClpA concentrations given are for the hexamer, and ClpP concentrations are for the 14-mer.

The mini-Mu plasmid used was pMK586 [34]. Recombination reactions were carried out in buffer A: 25 mM HEPES-KOH (pH 7.6), 140 mM NaCl, 15% (v/v) glycerol, 1 mM DTT, 10 mM $MgCl_2$, and 0.1 mg/ml BSA, unless otherwise noted. The ATP-mix stock solution included 80 mM ATP and a regeneration system of 0.5 mg/ml creatine kinase and 25 mM creatine phosphate. PD buffer contains 25 mM HEPES-KOH (pH 7.6), 5 mM KCl, 5 mM $MgCl_2$, 0.032% NP-40, 10% glycerol, 5 mM ATP, and an ATP regenerating system consisting of 16 mM creatine phosphate and 0.32 mg/ml creatine kinase.

Degradation Assays

Degradation was carried out as previously described [50]. Briefly, 1 μM MuA-ssrA, 1 μM GFP-ssrA, 0.3 μM ClpX, 0.8 μM ClpP, and 5 mM ATP-mix were incubated in PD buffer at 30°C. At the indicated times, samples were removed and stopped on ice with the addition of 0.2 volumes of 5× SDS-loading dye. Samples were separated by 8% SDS-PAGE. Degradation of MuA-ssrA was monitored by SYPRO-Orange staining (Molecular Probes).

STC/STC2 Purification

Standard 1× intramolecular strand transfer reactions contained MuA (3.2 pmol), HU (3.0 pmol), and donor DNA (1.3 pmol) in 25 μl buffer A. Assembly was conducted for 1 hr at 30°C. For purification of complexes by gel filtration, 50× assembly reactions were prepared (25 μl reaction volume, 160 pmol MuA, 150 pmol HU, 65 pmol donor DNA). STCs were challenged with 440 mM NaCl before injection onto a 1 ml column of BioGel A15m beads (BioRad) in buffer A without the BSA. Seventy-five microliter fractions were collected. STC2s were also purified over the BioGel A15m column. Ten microliters from each peak fraction was mixed with 0.2 volumes of SDS-sample buffer, boiled for 5 min, and run on a 10% SDS-PAGE. Immunoblotting was done as follows: protein was transferred to PVDF membrane (Immunoblon) by semidry transfer. Membrane was incubated with antibody to ClpX, anti-HRP, and finally ECF substrate. Membranes were then scanned on a FluorImager 595 (Molecular Dynamics).

Complex Destabilization Assays

Native gel electrophoresis assays were as previously described [20]. Briefly, ClpX or ClpA and ATP-mix were added to standard 1×

assembly reactions to final concentrations of 0.21 μM and 5 mM, respectively, and reactions were incubated at 30°C. Samples taken at specified times were stopped on ice, and 5 μl of each sample was run on a native 0.8% HGT agarose gel (FMC Bioproducts) in 1× TBA containing 80 μg/ml BSA (Fraction V, Sigma) and 10 μg/ml heparin (Sigma). Electrophoresis was for 1.75 hr at room temperature at 5.4 V/cm. Gels were stained for 20 min in a 1:10,000 dilution of Vistra Green.

Gel-Shift Assays

A 52 bp DNA fragment (PC fragment) with Mu binding sites was used for band shift assays and is described [51]. Labeled competitor (250 nM) was added with ClpX and ATP-mix to remodeling reactions. Samples taken at specified times were stopped on ice, and 5 μl of each sample was loaded on a 2% Metaphor agarose gel (FMC Bioproducts) in 0.5× TBE. Electrophoresis was for 2.5 hr at 4°C at 5.4 V/cm. Gels were pressed and dried on DEAE paper and either exposed to Kodak XAR film or to a Molecular Dynamics phosphorimaging cassette. Additionally, 5 μl of each sample was loaded and run on an HGT gel as described above.

Calculation of the released subunits was as follows: "total" release was measured using a mock sample in which all of the complexes were disrupted by salt treatment after ClpX remodeling. The samples were then diluted back to standard conditions for the binding experiment, and the percent of oligonucleotide shifted in this sample was quantitated and set to 100%. Similarly, samples incubated for 30 min without ClpX or without ATP were used to normalize for any spontaneous release.

Footprinting

Footprinting was essentially as described [24, 34, 40]. STCs were formed in 20× reactions, and assembly was stopped with 20 mM EDTA. Competitor (nonradioactive) oligonucleotide and ATP were added as for the gel-shift assay. ClpX and ATP were added to one sample to final concentrations of 1 μM and 5 mM respectively, while an equal volume of ClpX-storage buffer (and ATP mix) was added to the parallel sample. Remodeling was allowed to proceed for 30 min at 30°C, and reactions were stopped on ice. Gluteraldehyde was added to a final concentration of 0.08% and incubated at room temperature for 5 min, then quenched for 15 min at room temperature with 50 mM Tris (pH 8.0) and 10 mM lysine. Plasmid was digested with 10 U of either SwaI or EcoNI for 15 min at room temperature or 37°C, respectively. DNase (0.004 U) and 0.1 volumes of a 10 mM $MgCl_2$/5 mM $CaCl_2$ solution were added and incubated at room temperature for 1 min. Sucrose/EDTA stop buffer (0.2 volumes) was added immediately, and the reactions placed on ice. Samples were loaded and run by 5% PAGE. Bands at the wells, corresponding to the crosslinked complexes with synapsed ends, were excised and soaked overnight at 37°C in 100 mM ammonium acetate, 1% SDS, and 20 mM EDTA [34]. Samples were separated from acrylamide, EtOH precipitated, and resuspended in 10 mM Tris (pH 7.6). DNA products were then extended with primers to either the right or left end of the Mu genome (TB1406, 5′-GGCGTATCACGAGGCCCTT TCG-3′; TB1407, 5′-GCTGGAACCAGATGAAGCACGCC-3′). Primer extension products were loaded on a 6% Long Ranger (FMC Bioproducts) denaturing gel and electrophoresed at a constant 28 W. Gels were dried on DEAE paper and visualized by exposure to Kodak BAR film or to a phosphorimaging cassette.

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