Escherichia coli DNA Polymerase V Subunit Exchange

A POST-SOS MECHANISM TO CURTAIL ERROR-PRONE DNA SYNTHESIS*

Received for publication, September 11, 2003, and in revised form, October 21, 2003 Published, JBC Papers in Press, October 22, 2003, DOI 10.1074/jbc.M310127200

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DNA polymerase V consisting of a heterotrimer composed of one molecule of UmuC and two molecules of UmuD* (UmuD\(2C\)) is responsible for SOS damage-induced mutagenesis in Escherichia coli. Here we show that although the UmuD\(2C\) complex remains intact through multiple chromatographic steps, excess UmuD, the precursor to UmuD', displaces UmuD' from UmuD\(2C\) by forming a UmuDD\(_2\)-heterodimer, while UmuC concomitantly aggregates as an insoluble precipitate. Although soluble UmuD\(2C\) is readily detected when the two genes are co-transcribed and translated in vitro, soluble UmuD\(_2\)C or UmuDD\(_2\)C are not detected. The subunit exchange between UmuD\(2C\) and UmuD offers a biological means to inactivate error-prone polymerase V following translesion synthesis, thus preventing mutations from occurring on undamaged DNA.

The Escherichia coli SOS response was first described in the mid-1970s (1, 2) and is now known to involve the induction of >40 genes under control of the LexA repressor protein (3). Although many of the genes in the SOS regulon are involved in DNA damage repair and cell division (4), two “UV mutagenesis” genes, umuC and umuD (5–7), are required to observe either UV- or chemically induced mutations elevated typically ~100-fold above spontaneous background levels (4). SOS-induction mutations on the bacterial chromosome result primarily from the error-prone replication of damaged DNA templates by pol V,1 a heterotrimer composed of one UmuC molecule bound to two molecules of UmuD* (UmuD\(2C\)) (8, 9).

Based upon the inherent error-prone nature of TLS, it is perhaps not too surprising that the activity of the Umu proteins is regulated at transcriptional and post-translational levels (10). The umu genes are arranged in an operon and are negatively regulated at the transcriptional level by the SOS repressor LexA (7). Despite the fact that the umu operon is one of the tightest regulated in the LexA regulon (11), transcriptional regulation is incomplete, and the cellular levels of UmuD and UmuC are additionally kept to a minimum through their rapid Lon-mediated proteolytic degradation (12, 13). After cellular DNA damage, RecA nucleates on regions of single-stranded DNA and mediates the post-translational self- cleavage of LexA leading to its inactivation and derepression of genes in the LexA regulon, including umuD and umuC (4). UmuD undergoes a mechanistically similar cleavage reaction (14, 15). In the case of UmuD, cleavage of its N-terminal 24 amino acids converts it to UmuD' and activates it for its role in SOS mutagenesis (16). UmuD and UmuD' both form homodimers but when mixed together associate preferentially as a UmuDD' heterodimer (17), and UmuD' becomes susceptible to proteolysis by the ClpXP serine protease (12, 18). Degradation of the mutagenically active UmuD' subunit helps return cells to a resting state once cellular DNA damage has been repaired and the need for pol V has abated.

In vitro replication assays reveal that regulation of the catalytic activity of pol V is also complex and is modulated through multiple protein-protein interactions. For example, pol V does not catalyze TLS alone but is, instead, an essential component of a multiprotein “mutasome” complex composed of RecA protein, β sliding-clamp, and single-strand binding protein (19, 20). When taken in conjunction with the observation that pol V is, for all intents and purposes, catalytically inactive in the absence of UmuD' or RecA (21, 22), previous studies have understandably focused almost exclusively on the biochemical properties of pol V when working as part of a mutasomal complex during TLS (21–24). Here, we investigated the in vitro stability of the UmuD\(2C\) complex and its ability to undergo subunit exchange with UmuD and UmuD' proteins. Although a soluble UmuD\(2C\) complex can be purified over multiple chromatographic steps, UmuD* is readily displaced from UmuC by UmuD, concomitantly causing UmuC to aggregate as an inactive insoluble precipitate. Subunit exchange between UmuD\(_2\)C and UmuD provides a biologically plausible way to curtail the activity of error-prone pol V following TLS and thus reduces the chance that mutations will occur on undamaged DNA during SOS.

EXPERIMENTAL PROCEDURES

Materials—Ultrapure deoxyribonucleotides were purchased from Amersham Biosciences. ATP[S] was purchased from Roche Applied Science. RecA protein was purified as described previously (25). E. coli DNA pol V (UmuD\(2C\)) was purified as a native untagged complex (9, 26) from E. coli strain RW630, a ΔpolB ΔumuDC ΔdinB K-12 strain. Wild type UmuD, UmuD1 (P27S) mutant, and UmuD' were purified following protocols described previously (8, 27, 28). The in vitro transcription/translation kit (E. coli T7 S30 extract system for circular DNA) was purchased from Promega.

Interaction between pol V and in Vitro [\(^35\)S]-Labeled UmuD Protein—Purified UmuD protein was labeled in vitro following a protocol modified from previous studies (29, 30). In the labeling reaction, UmuD (5 mg/ml) and Trans \(^35\)S-labeled mix from ICN Biomedicals (0.5 μCi/ml) were incubated in 20 mM Tris-HCl (pH 8.5) and 10 mM EDTA at 37 °C for 2 h. The mixture was loaded first to a Bio-spin P-6 column from Bio-Rad and then to a 20-ml Sephadex G-75 column to isolate the

* This work was supported by National Institutes of Health Grants GM42554 and GM21422 (to M. F. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: pol V, polymerase V; TLS, translesion synthesis; ATP[S], adenosine 5'-3-O-(thio)triphosphate.

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Polymerase V Inactivation by UmuD Protein

35S-labeled proteins. Fractions containing labeled UmuD2 homodimer were collected. Unlabeled pol V (UmuD') complex was mixed with 35S-labeled UmuD2 homodimer in a 1:3 ratio (0.7 μM and 2.1 μM, respectively) in R buffer (20 mM Tris·HCl, pH 7.5, 0.1 mM EDTA, 1 mM dithiothreitol, 20% glycerol) with 300 mM NaCl and incubated at 37 °C for different lengths of time. Samples were chilled on ice before centrifugation at 14,000 × g in a microcentrifuge for 10 min. The soluble supernatant was then separated on a 20-ml Superdex G-75 column at 4 °C. The radioactivity from each gel filtration fraction was measured on a liquid scintillation counter, and SDS-PAGE was used to determine the distribution of 35S-labeled UmuD and unlabeled pol V subunits. A control experiment was carried out with 35S-labeled UmuD homodimer incubated for 1 h at 37 °C.

Polymerase V Catalyzed Primer Extension and Translesion Synthesis An assay—A synthetic 30-nucleotide primer (5′-ACT GAC CCC GTT AAA ACT TAT TAC CAG TAA-3′) was 5′-labeled with 32P and annealed to a 39-nucleotide template (5′-TAC GGT TTA CTG GTA ATA AGT TTT AAC GGG GTC AGT-3′) containing an abasic site (X), generating a 9-nucleotide overhang downstream of the primer 3′-end. The abasic site (X) on the template was 3 nucleotides from the primer 3′-end. The reaction buffer contained 20 mM Tris·HCl (pH 7.5), 8 mM MgCl2, 5 mM dithiothreitol, 0.1 mM EDTA, 25 mg/ml bovine serum albumin and 4% (v/v) glycerol. Polymerase V (400 nM) was either pre-incubated at 37 °C alone for 3 min or with purified UmuD, UmuD1, or UmuD' proteins before being added to a mixture of DNA (10 nm), RecA (250 nm), ATP-s (1 mM), and 4 × NDEPS (200 μM each) to initiate the reaction. The reaction was incubated at 37 °C for 10 min and quenched by adding an equal volume of 95% formamide, 40 mM EDTA. The products were heat-denatured and separated on denaturing polyacrylamide gels. Radioactive gel bands were analyzed on a PhosphorImager using ImageQuant software (Amer sham Biosciences).

Cross-linking Study of Interaction between pol V and UmuD—The method used for cross-linking experiments was adapted from Ref. 31. Polymerase V, UmuD, UmuD', and bovine serum albumin were incubated in different combinations at 37 °C for 10 min in a buffer containing 50 mM HEPES, pH 7.5, 100 mM potassium glutamate, 10 mM MgCl2, and 1 mM dithiothreitol before formaldehyde was added to 1% to initiate cross-linking. The mixture was incubated for 20 min at room temperature following the addition of formaldehyde and quenched by SDS-PAGE loading buffer. The samples were heated in a boiling water bath for 5 min and resolved on a 14% SDS-PAGE. Western blotting was conducted with antibodies that react with UmuDD fusion protein (24). Aside from the observation that pol V can be purified as a UmuD'2C heterotrimer (9), little is known about the physical properties of the heterotrimeric complex.

An important aspect regarding the regulation of pol V is to determine the ability of pol V to exchange the UmuD' subunits bound to UmuC. Using 35S-labeled UmuD, we measured the exchange of the UmuD' subunit in pol V by the mutagenically inactive UmuD subunit (Fig. 1). Purified pol V and [35S]UmuD (present primarily in the form of a dimer, i.e. [35S]UmuD2) were incubated for 1, 10, and 60 min, and the products were resolved by gel filtration with Superdex G75. Both peaks containing [35S] are present in the G75-included volume (Fig. 1a). There is a decreasing amount of [35S] label co-eluting with the UmuD2 or UmuDD' dimer peak with increasing incubation time (Fig. 1a, fractions c and d) and an increasing amount of [35S] label with the UmuD monomer peak (Fig. 1a, fraction g), demonstrating the loss of input [35S]UmuD2 homodimer caused by the replacement of [35S]UmuD with UmuD' to form [35S]UmuDD' heterodimers. The presence of increasing levels of [35S]UmuD at 10 min and 1 h imply that the concentration of released monomeric [35S]UmuD is too dilute to re-form a [35S]UmuD2 homodimer.

The identity of the product in each peak was determined using Coomassie Brilliant Blue-stained gels (Fig. 1b). The “heavy” peaks (Fig. 1b, fractions c and d) corresponding to a 10-min incubation contain both UmuD and UmuD'. The heavy peaks elute with a molecular mass consistent with a UmuDD' heterodimer complex (Fig. 1a). The “light” peak (Fig. 1b, fraction g) is composed primarily of UmuD and contains a trace amount of UmuD' (Fig. 1b); this peak elutes with molecular mass markers corresponding to monomer species (Fig. 1b). As a control, [35S]UmuD was incubated in the absence of pol V for 60 min and remained intact as a dimer (Fig. 1a, inset). Absent from

RESULTS AND DISCUSSION

Subunit Swapping of pol V with 35S-Labeled UmuD Protein—We have purified pol V as a native heterotrimeric complex (UmuD'C) that remains intact and stable in aqueous solution (9). In contrast, UmuC containing the polymerase active site (21, 24) is essentially insoluble in aqueous solution (8, 9, 24) unless expressed as a recombinant maltose-binding fusion protein (24). Aside from the observation that pol V can

FIG. 1. Exchange of pol V subunits with 35S-labeled UmuD proteins. a, pol V was incubated with 35S-labeled UmuD protein, and the exchange products were resolved by Sephadex G-75 gel filtration. The level of radioactivity in each fraction was measured by liquid scintillation counting and plotted with decreasing molecular mass from left to right. The times of incubation were: 1 min, 10 min, and 1 h shown as closed circles, inverted triangles, and squares, respectively. The molecular ratio of UmuD to pol V was 3:1. Inset, 35S-labeled UmuD incubated for 1 h in the absence of pol V. b, representative fractions (a–g, from panel a) chromatographed on SDS-PAGE. The protein gel was stained with Coomassie Brilliant Blue. UmuD and pol V protein markers are shown at the left of the gel. M designates molecular mass markers.
any of the gel elution fractions, including the void volume, were UmuD\textsubscript{2}C or UmuDD\textsubscript{2}C, whereas “free” UmuC was detected as an insoluble aggregate contained in the centrifuged pellet of the UmuD\textsubscript{2}C/\textsuperscript{35}S[UmuD\textsubscript{2}], exchange reaction by Western blotting against the protein resolubilized in 8 M urea (data not shown).

**Polymerase V Is Stabilized by UmuD and Destabilized by UmuD**—The gel filtration data demonstrate the important point that the UmuD subunits of the pol V heterotrimer in solution are exchangeable with free UmuD or UmuD\textsubscript{2}, resulting in the formation of UmuDD\textsubscript{2} while releasing UmuC as an insoluble precipitate. The corollary to this observation is that incubation of UmuD with pol V should inactivate the polymerase, because the catalytic activity is present only in UmuC (21, 24). The ability of pol V to copy undamaged DNA and to catalyze TLS is reduced in the presence of UmuD, the inhibition being much stronger at higher concentrations of UmuD (Fig. 2). A similar result occurred when pol V was incubated with UmuD1, a noncleavable UmuD mutant that cannot be converted to UmuD\textsuperscript{2} in the presence of activated RecA (32) (Fig. 2).

In contrast, a small but significant (2-fold) stimulation of pol V activity occurs in the presence of excess UmuD\textsuperscript{2} (Fig. 2). Thus, although the UmuD\textsubscript{2}C complex is relatively stable and is able to maintain its integrity through multiple purification stages (9, 21), the exchange of free UmuD or UmuD\textsuperscript{2} proteins with a UmuD\textsuperscript{2} monomeric component of pol V (UmuD\textsubscript{2}C), nevertheless, takes place. Stimulation of pol V could occur if the inactive UmuD\textsubscript{2}C is converted to active UmuD\textsuperscript{2}C in the presence of excess UmuD. Our data, therefore, imply the existence of an equilibrium between UmuD\textsubscript{2}C and UmuD\textsuperscript{2}C that strongly favors UmuD\textsubscript{2}C.

**Identification of pol V Subunit Exchange Products by Formaldehyde Cross-linking Analysis**—Combined formaldehyde cross-linking and Western blot analyses were performed to identify the complexed and free forms of the Umu proteins following the addition of excess UmuD or UmuD\textsuperscript{2} to pol V (Fig. 3). Incubation of UmuD alone followed by cross-linking with formaldehyde resulted in the formation of a UmuD\textsubscript{2} complex and free UmuD (Fig. 3, lane 4), and a similar experiment using UmuD\textsuperscript{2} resulted in formation of UmuD\textsubscript{2} and free UmuD (Fig. 3, lane 5). Co-incubation of UmuD and UmuD\textsuperscript{2} followed by cross-linking of the products resulted in the appearance of one additional complex of UmuDD\textsuperscript{2} (Fig. 3, lane 6).

In a parallel experiment using formaldehyde cross-linking, when increasing amounts of UmuD were incubated with a fixed amount of pol V, the exchange product was UmuDD\textsuperscript{2} (Fig. 3, lanes 8–10). That is, essentially all of the UmuD\textsubscript{2}C (Fig. 3, lane 7) was converted to a UmuDD\textsuperscript{2} plus UmuC, and no soluble formaldehyde cross-linked UmuD\textsubscript{2}C or UmuDD\textsubscript{2}C complexes were observed. The absence of detectable free UmuC by Western analysis (Fig. 3, lanes 8–10) is attributable to its insolubility in aqueous solution (8, 9, 24, 33) causing it to aggregate and form a visible precipitate. However, a clear UmuC signal was observed on a Western blot of pol V incubated in the absence of UmuD (Fig. 3, lane 7).

The addition of increasing amounts of UmuD to pol V resulted in the recovery of only pol V (where both UmuC and UmuD\textsuperscript{2} are detected) + free UmuD\textsuperscript{2} (Fig. 3, lanes 11–13). There was a roughly 1.5–2.0-fold increase in the intensity of the UmuD\textsubscript{2}C band incubated in the presence of excess UmuD\textsuperscript{2} (Fig. 3, lanes 11–13) compared with no incubation with UmuD\textsuperscript{2} (Fig. 3, lane 7). These data are consistent with those showing that an excess UmuD\textsuperscript{2} stimulates pol V activity by a similar 1.5–2-fold increase (Fig. 2). There was no UmuDD\textsuperscript{2} exchange observed when proteins were cross-linked with formaldehyde prior to the protein co-incubation reactions (Fig. 3, lanes 17–19). Once again, our data imply that there is ongoing subunit exchange within pol V and that formaldehyde cross-linking helps minimize the exchange and the aggregation of insoluble UmuC.

**Transcription/Translation In Vitro Synthesis of pol V to Identify Putative Umu Complexes**—Although it is tempting to suggest that soluble complexes composed of UmuD\textsubscript{2}C do not occur based on the inability to detect them by swapping UmuD and UmuC subunits using highly purified proteins (Figs. 1 and 3), it is nevertheless possible that such complexes can still form in vivo. Perhaps the best chance of mimicking “life-like” conditions in vitro is to use a transcription/translation system to co-express \textsuperscript{35}S-labeled UmuC and UmuD proteins (Fig. 4). Each
of the overexpressed $^{35}$S-labeled protein products, UmuC, UmuD, and UmuD', were readily detected by SDS-PAGE (Fig. 4a) or by SDS-PAGE following gel filtration on Superdex G200 (Fig. 4, b-f). UmuD' and UmuC gave products of the expected mass. In vitro transcription/translation of UmuD gave two products, one that corresponds to full-length UmuD and one that migrates slightly faster than UmuD'. The expression of this smaller protein was variable and was even evident in a noncleavable mutant of UmuD, suggesting that its expression was driven from the UmuD plasmid itself and not from post-translational modification of UmuD. Thus, pol V-dependent mutagenesis on undamaged DNA to a minimum.

![Fig. 4. Umu complexes formed using coupled transcription/translation.](image)

**Fig. 4.** Umu complexes formed using coupled transcription/translation. Plasmids containing genes for the expression of UmuC, UmuD, and UmuD' were transcribed and translated as described under “Experimental Procedures.” a, SDS-PAGE of $^{35}$S-labeled in vitro transcription/translation products. C, D, and D' to the left indicate the gel mobilities of UmuC, UmuD, and UmuD', respectively. b-f, $^{35}$S-labeled in vitro transcription/translation products were separated by Superdex G-200 gel filtration, and each fraction was resolved by SDS-PAGE. The identity of each plasmid is shown at the top of the gels. The fractions containing UmuD3, UmuD2, or UmuC are indicated at the left of the gels; the fraction containing UmuD'C (pol V) is present in panel e, indicated at the bottom of the gel. MW, molecular weight.

insoluble protein aggregates and is removed by centrifugation prior to loading the sample on the column. A key difference in the two gel filtration experiments is that UmuC is present in $\mu$g amounts when carrying out subunit exchange using purified proteins (Fig. 1) but only in ng amounts using the transcription/translation system (Fig. 4).

We also carried out similar in vitro co-expression experiments with noncleavable UmuD1 in place of wild type UmuD, and the results obtained were similar to that seen with wild type UmuD (data not shown). In an experiment where UmuC, UmuD1, and UmuD' were all co-expressed together, most of the UmuD1 and UmuD' migrated as a soluble heterodimer, whereas UmuC and a small fraction of UmuD1D' eluted in the void volume as insoluble aggregates.

**Biological Relevance of pol V Subunit Exchange**—Based upon the known biochemical properties of the Umu proteins, it has been hypothesized that they form a variety of protein complexes. UmuD and UmuD' can exist as homodimers but prefer to heterodimerize whenever possible. UmuC may exist as a monomer as well as in a complex with UmuD2, UmuD'D', and UmuD'. Although the UmuD and UmuD' proteins have been successfully isolated in both homodimeric (8, 15) and heterodimeric forms (8), UmuC has only been isolated in significant quantities as a soluble UmuD'C complex (9, 21, 23), thereby raising the question of whether UmuC, UmuD2, or UmuD'D'C are present in vivo. And, if present in vivo, they may still be largely insoluble in the cell.

To date, monomeric UmuC has only been isolated as a recombinant maltose-binding protein fusion (24) or as a native untagged form but only after denaturation and renaturation of the overexpressed protein (8, 33). A key step in the purification of denatured/renatured UmuC included affinity chromatography utilizing UmuD'D' proteins as the ligand. UmuC bound avidly to this column and could only be eluted under denaturing conditions (8). These observations were originally interpreted as indicating tight complex formation between UmuD'D' and UmuC. However, given our inability to detect any such complexes in solution, we now believe it much more likely that

![Fig. 5. Regulation of pol V via subunit exchange.](image)

**Fig. 5.** Regulation of pol V via subunit exchange. Once cellular DNA damage has been repaired, the SOS-inducing signal wanes, and there is a concomitant decrease in the rate at which UmuD is converted to UmuD'. Although UmuD can exist as a homodimer, it prefers to heterodimerize with UmuD' and, in doing so, displaces UmuC, causing it to aggregate as an insoluble and inactive precipitate. When complexed with UmuD, UmuD' becomes a substrate of the ClpXP protease and is rapidly degraded. This allows UmuD to homodimerize and leads to the Lon-mediated proteolytic degradation of UmuD. Thus, pol V subunit exchange combines with efficient proteolytic degradation of the Umu proteins helps to curtail error-prone translesion DNA synthesis and keep pol V-dependent mutagenesis on undamaged DNA to a minimum.
UmuDD' caused UmuC to aggregate and precipitate on the column.

In vivo, co-expression of UmuDD' and UmuC also led to a dramatic increase in the half-life of UmuC (34). This observation was also taken as an indication that UmuDD' interacts with UmuC so as to protect it from Lon-mediated degradation. Perhaps a more likely alternative hypothesis, based on our current data, is that the apparent increase in the stability of UmuC resulted from interactions between UmuDD' and UmuC that caused UmuC to aggregate within the cell, thereby making it inaccessible to Lon-proteolysis. The in vivo and in vitro data taken together offer convincing evidence that any interaction between UmuC and UmuD2 or UmuDD' appears quantitatively and qualitatively different from that involving UmuD'C. The latter is stable and resistant to cellular proteolysis and is readily purified as a complex (9, 21, 23). Even so, the pol V complex is susceptible to subunit exchange.

We propose that subunit exchange provides an internal “self-destruct” mechanism to curtail the activity of error-prone pol V following TLS, and this reduces the chance that mutations will occur on undamaged DNA during SOS. Our data show that subunit exchange between the UmuD'c complex and intact UmuD has two dramatic effects. First, it most likely leads to the inactivation of the catalytic function of UmuC as the protein aggregates as an insoluble precipitate (Fig. 5). Second, it results in the formation of a soluble UmuD'c complex in which UmuD' is susceptible to rapid proteolysis by ClpXP (Fig. 5). It is possible that monomeric UmuC and UmuD2C exist in a cell, but the physical properties of such Umu complexes appear to be entirely different from that of the soluble and catalytically active UmuD'C (pol V). Thus, based on current biochemical data, we invoke Occam’s razor to suggest that the sole soluble complex involving UmuC in the cell is a heterotrimer composed of UmuD'C, which is very likely the biologically relevant form of pol V (20).

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