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τ Binds and Organizes *Escherichia coli* Replication Proteins through Distinct Domains

DOMAIN III, SHARED BY γ AND τ, OLIGOMERIZES DnaX*

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Bradley P. Glover‡, Arthur E. Pritchard, and Charles S. McHenry§

From the Department of Biochemistry and Molecular Genetics, University of Colorado Health Sciences Center, Denver, Colorado 80262

The τ and γ proteins of the DNA polymerase III holoenzyme DnaX complex are products of the *dnaX* gene with γ being a truncated version of τ arising from ribosomal frameshifting. τ is comprised of five structural domains, the first three of which are shared by γ (Gao, D., and McHenry, C. (2001) *J. Biol. Chem.* 276, 4433–4453). In the absence of the other holoenzyme subunits, DnaX exists as a tetramer. Association of δ, δ′, χ, and ψ with domain III of $DnaX_4$ results in a DnaX complex with a stoichiometry of $DnaX_3\delta\delta'\chi\psi$. To identify which domain facilitates DnaX self-association, we examined the properties of purified biotin-tagged DnaX fusion proteins containing domains I-II or III-V. Unlike domain I-II, treatment of domain III-V, γ, and τ with the chemical cross-linking reagent BS3 resulted in the appearance of high molecular weight intramolecular cross-linked protein. Gel filtration of domains I-II and III-V demonstrated that domain I-II was monomeric, and domain III-V was an oligomer. Biotin-tagged domain III-V, and not domain I-II, was able to form a mixed DnaX complex by recruiting τ, δ, δ′, χ, and ψ onto streptavidin-agarose beads. Thus, domain III not only contains the δ, δ′, χ, and ψ binding interface, but also the region that enables DnaX to oligomerize.

The DNA polymerase III holoenzyme is the primary replicative polymerase of *Escherichia coli* comprising 10 subunits: α, τ, γ, β, δ, δ′, ϵ, ψ, χ, and θ. These proteins assemble and function as three distinct subassemblies: (i) the DNA polymerase Pol III core, (ii) the ring-shaped processivity factor β, and (iii) the DnaX complex ($\tau_2\gamma_1\delta\delta'\chi\psi$) that assembles β onto primed templates and plays an important role in the organization of the replication fork (reviewed in Refs. 1 and 2). The DnaX complex clamp loader contains one set of the auxiliary subunits, which impart an overall structural asymmetry to the replicase and bind to γ within native holoenzyme (3–6). δ and δ′ form a 1:1 complex and function with DnaX to load β onto primed templates in an ATP-dependent manner (7, 8). The χψ subunits bind single-stranded DNA-binding protein and play a role in lagging strand initiation complex formation (9–11).

The τ and γ proteins present within the DnaX complex are both products of the *dnaX* gene with γ being a truncated version of τ arising from programmed ribosomal frameshifting (12–17). We have demonstrated that DnaX organizes the holoenzyme by binding replication proteins through five distinct structural domains (18–22). The portion of DnaX common to both τ and γ contains the ATPase active site (domain I) and is responsible for binding the auxiliary subunits δ, δ′, χ, and ψ (domain III) (20). Domains IV and V make up the C-terminal portion of τ and allow the full-length DnaX gene product to interact with the DnaB helicase and core polymerase, respectively, facilitating leading and lagging strand synthesis at the replication fork (19, 20, 23–26, 42, 43).

DnaX is a homotetramer[1] in the absence of the other polymerase proteins. We have demonstrated that the association of the auxiliary subunits δ, δ′, χ, and ψ with $DnaX_4$ results in a DnaX complex with an overall stoichiometry of $DnaX_3\delta_1\delta'_1\chi_1\psi_1$ (29). The DnaX complex formation-mediated transition of $DnaX_4$ to $DnaX_3$ occurs for DnaX complexes containing either or both τ and γ. The portion of DnaX present in τ, and absent in γ (domains IV and V), has recently been shown to be a monomeric (26). Thus, the N-terminal 430 residues common to both τ and γ not only have the minimal protein sequence necessary to bind the auxiliary subunits δ, δ′, χ, and ψ, and load the β processivity factor onto a primed template in an ATP-dependent manner, but also the sequence required to oligomerize DnaX protomers. The binding of the auxiliary subunits to domain III influences the oligomerization state of DnaX during DnaX complex assembly, suggesting that domain III could provide the protein-protein interface responsible for DnaX oligomerization. The focus of this report is to determine which structural domain contained within the portion of DnaX common to both γ and τ (domains I-III) is responsible for the oligomerization of the DnaX protein.

EXPERIMENTAL PROCEDURES

Proteins, Nucleic Acids, and Reagents—DNA polymerase III holoenzyme protein subunits were purified according to the references given:

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‡ Present address: Dept. of Molecular and Cell Biology, University of California, Berkeley, CA 94720.

§ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Genetics, University of Colorado Health Sciences Center, 4200 East Ninth Ave., B121, Denver, CO 80262.

[1] The abbreviations used are: homotetramer, a DnaX assembly containing four τ or γ protomers; heterotetramer, a DnaX assembly containing both τ and γ present in an overall DnaX stoichiometry of four; homooligomer, an oligomeric form of DnaX containing more than one τ or γ protomer; heterooligomer, an oligomeric form of DnaX containing at least one τ and γ protomer; mixed or heteromeric DnaX complex, a DnaX complex containing both τ and γ and the δ, δ′, χ, and ψ proteins; auxiliary subunits, refers to δ, δ′, χ, and ψ; NΔ221τ, an N-terminal biotin and hexahistidine-tagged τ protein with the N-terminal 221 amino acids deleted; domain III-V, NΔ221τ that contains τ domain III-V; CΔ422τ, a C-terminal biotin and hexahistidine-tagged τ protein with the C-terminal 422 amino acids deleted; domain I-II, CΔ422τ that contains τ domains I-II; BS3, [bis(sulfosuccinimidyl)suberate]; PAGE, polyacrylamide gel electrophoresis; NTA, nitrilotriacetic acid; FPLC, fast protein liquid chromatography.

Pol III core (30), β (31), τ and γ (32), δ and δ′ (33), χψ (34), NΔ221τ (domain III-V) and CΔ422τ (domain I-II) (20), DnaG primase and single-stranded DNA-binding protein (35). Yeast extract (Ardamine Z) and tryptone used in the fermentor growth of bacteria for protein overexpression were from Universal Flavors (Juneau, WI) and DMV International Nutrition (Fraser, NY), respectively. [^{3}H]Deoxythymidine 5′-triphosphate was purchased from ICN. Nonlabeled nucleotides were purchased from Amersham Pharmacia Biotech. BS3 was from Pierce.

BS3 Cross-linking and Immunoblotting of DnaX Proteins—BS3 stock solutions were prepared fresh before every reaction by dissolving BS3 in buffer B (50 mM HEPES (pH 7.4), 5% glycerol, and 50 mM NaCl). An aliquot of the BS3 stock solution was added to the protein immediately and reacted at room temperature for 40 min. Reactions were quenched by the addition of SDS-PAGE sample buffer. Domain I-II, domain III-V, τ, and γ were cross-linked with 10 μM BS3.

Proteins were loaded onto a 10% SDS-polyacrylamide gel and separated at 25 mA for 2.5 h. The separated proteins were electrotransferred to Immobilon-P polyvinylidene difluoride membrane at 500 mA for 6 h and blocked in MTBS (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5% nonfat milk) overnight at 4 °C. Membrane containing lanes of biotin-tagged domain I-II and domain III-V were biotin-blotted by incubating the membrane with horseradish peroxidase-conjugated streptavidin (1:1000 dilution in MTBS) and developed with the enhanced chemiluminescent (ECL) method (Amersham Pharmacia Biotech). Membranes containing lanes of τ and γ were immunoblotted with DnaX-specific antibody 527G1 (1:1000 dilution in MTBS). Immunostaining was visualized using a biotinylated secondary anti-mouse antibody (1:1000 dilution in MTBS) followed by horseradish peroxidase-conjugated streptavidin (1:1000 dilution in MTBS) and developed with the enhanced chemiluminescent method. Membranes were washed in TBST (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 20) following incubations with the primary antibody, secondary antibody, and the horseradish peroxidase-conjugated streptavidin (1 × 15 min and 2 × 5 min).

Stokes Radius Determination by Gel Filtration—A 24-ml Superose 12 FPLC gel filtration column (Amersham Pharmacia Biotech) equilibrated in buffer G (50 mM HEPES (pH 7.5), 250 mM sodium chloride and 5% glycerol) was calibrated with high and low molecular weight gel filtration calibration kits (Amersham Pharmacia Biotech). Blue dextran (A_{600} = 650), thyroglobulin (630 μg), ferritin (500 μg), catalase (400 μg), aldolase (200 μg), albumin (500 μg), ovalbumin (500 μg), chymotrypsinogen A (400 μg), ribonuclease A (500 μg), τ (690 μg), γ (630 μg), domain III-V (445 μg), and domain I-II (155 μg) were dissolved in buffer G and loaded (250-μl injection volume) onto the Superose 12 column and developed in buffer G in separate experiments. Gel filtration fractions (0.5 ml) were collected at 4 °C in buffer G with a flow rate of 0.3 ml/min. Proteins were identified in eluted fractions using the Pierce Coomassie Plus protein assay. Bovine serum albumin (fat-free; Sigma) was used as a protein assay standard. Superose 12 fractions containing NΔ221τ and CΔ422τ were subjected to 10% SDS-polyacrylamide gel electrophoresis at 25 mA for 2.5 h. Subsequently, gels were stained with Coomassie Brilliant Blue R-250 in 20% methanol and 10% acetic acid with destaining being done in 10% methanol and 10% acetic acid. The elution position of blue dextran was used as the Superose 12 void volume.

Heteromeric DnaX Complex Formation with Domain I-II and Domain III-V on Streptavidin-Agarose Beads—The potential for domain I-II or domain III-V to form heterooligomeric DnaX complexes with intact τ was tested by *in vitro* incubation followed by a streptavidin bead procedure described elsewhere (36). Either domain I-II or domain III-V (0.17 nmol) was incubated at 15 °C with τ (12.6 μg, 0.17 nmol) in a volume made up to 20 μl with buffer S (20 mM Tris (pH 7.5), 25 mM NaCl, 0.1 mM EDTA, 20% (w/v) glycerol, 5 mM dithiothreitol) and NaCl added to a final concentration of 90 mM. After 2 h the incubation mixture was added to an 11.2-μl volume on ice containing δ′ (8.25 μg, 0.22 nmol), δ (8.6 μg, 0.22 nmol), and χψ (6.9 μg, 0.22 nmol). The mixtures were flash-frozen and stored at −70 °C prior to the streptavidin bead procedure, which purifies biotinylated proteins and their associated complexes away from nonbiotinylated proteins (36). The purified complexes were removed from the beads by boiling in an SDS-PAGE loading buffer and then analyzed by gel electrophoresis.

RESULTS

Expression and Purification of the Truncated DnaX Fusion Proteins Containing Domain I-II and Domain III—We utilized two plasmids, each encoding specific structural domains of τ (Fig. 1*A*) under control of an inducible promoter described previously (20). Plasmid P_{A1}-NΔ221τ encoded protein NΔ221τ (domain III-V). This protein has 221 amino acids deleted from the full-length τ's N terminus with a fusion peptide added to the new N terminus. The plasmid pET11-CΔ422τ encoded protein CΔ422τ (domain I-II). This protein has 422 amino acids deleted from the full-length τ's C terminus with a fusion peptide added to the new C terminus. Each of these proteins possessed a fusion peptide that contained a hexahistidine sequence allowing purification via Ni^{2+}-NTA metal affinity chromatography, and a short biotinylation sequence facilitating their detection by biotin blotting. Both domain III-V (Fig. 1*B*, *lanes 1–3*) and domain I-II (Fig. 1*C*, *lanes 1–3*) were purified to >85% purity as determined by Coomassie-stained SDS-PAGE gels. Biotin blotting demonstrated that domain III-V (Fig. 1*B*, *lane 6*) and domain I-II (1*C*, *lane 6*) were the only biotinylated proteins present in the final purified preparation. Neither domain III-V nor domain I-II possess the ability to reconstitute DNA polymerase activity due to the absence of the δδ′χψ binding domain (domain III) and ATPase active site, respectively.

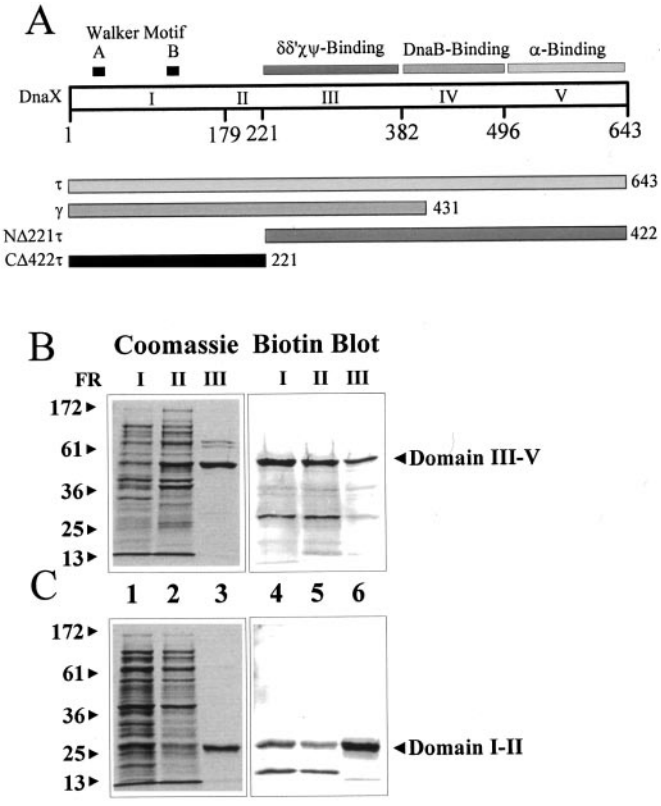

FIG. 1. **Purification of domain I-II and domain III-V.** *A*, wild-type τ contains domains I-V, wild-type γ I-III, biotin-tagged NΔ221τ domains III-V, and biotin-tagged CΔ422τ domains I and II. *B*, Coomassie-stained SDS-PAGE gel (*lanes 1–3*) and biotin blot (*lanes 4–6*) of the purification of domain III-V. *Lanes 1* and *4*, fraction I (*FR I*) cell lysate (10 μg); *lanes 2* and *5*, fraction II (*FR II*) ammonium sulfate (10 μg); and *lanes 3* and *6*, fraction III (*FR III*) Ni^{2+}-NTA purification (3 μg). *C*, Coomassie (*lanes 1–3*) and biotin blot (*lanes 4–6*) of the purification of domain I-II. *Lanes 1* and *4*, fraction I cell lysate (10 μg); *lanes 2* and *5*, fraction II ammonium sulfate (10 μg); and *lanes 3* and *6*, Ni^{2+}-NTA purification (3 μg).

BS3 Cross-links DnaX Proteins Containing Domain III—Chemical cross-linking is a well established method allowing for the identification of protein-protein interactions within multisubunit complexes (37–39). Previously we employed the homobifunctional cross-linking reagent BS3 in demonstrating that the auxiliary subunits δ, δ′, χ, and ψ bind to γ, and not τ, within authentic holoenzyme (6). We found that treatment of DnaX or the DnaX complex with BS3 produced covalent cross-links between proteins within the same complex. We used BS3

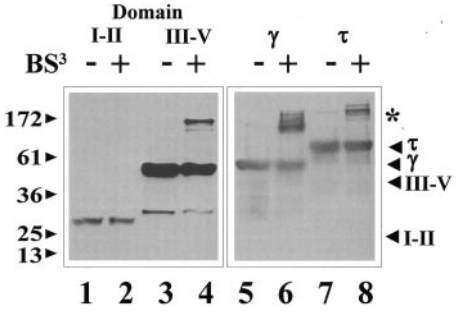

FIG. 2. **BS3 cross-links DnaX proteins containing domain III.** Domain I-II (*lanes 1* and *2*, 12 μg each lane), domain III-V (*lanes 3* and *4*, 12 μg each lane), γ (*lanes 5* and *6*, 8 μg each lane), and τ (*lanes 7* and *8*, 8 μg each lane) were cross-linked at room temperature for 40 min with 10 μM BS3, quenched, subjected to 4–15% SDS-PAGE, and biotin-blotted (for domain I-II and domain III-V) or immunoblotted (for γ and τ) with an anti-γ monoclonal antibody. The *star* indicates high molecular mass cross-linked protein bands.

as a tool to determine whether domain I-II and domain III-V formed homooligomers.

We treated DnaX domains I-II, III-V, γ, and τ with 10 μM BS3. Reaction products were visualized by biotin blotting (domain I-II and domain III-V) and Western blotting with a DnaX-specific monoclonal antibody (for γ and τ). No high molecular weight cross-link bands were evident in reactions containing domain I-II (Fig. 2, compare *lanes 1* and *2*). A distinct band that migrates as a 159-kDa protein relative to markers was observed in domain III-V BS3 cross-linking reaction (compare *lanes 3* and *4*). The predicted molecular weights for a dimer, trimer, and tetramer of domain III-V are 100, 150, and 200 kDa, respectively. Because only one high molecular weight cross-link band is observed with no intermediate, smaller bands being apparent, the 150-kDa species is probably a dimer of domain III-V. Cross-linked proteins joined near their termini would be expected to migrate with a molecular weight consistent with the additive weights of the their components. Proteins within the same complex, which cross-link at internal positions to each other, exhibit apparent molecular weights larger than the additive weights of the complex components. These "X-shaped" cross-linked protein complexes migrate more slowly through the SDS-polyacrylamide matrix during electrophoresis. Thus, our observation is consistent with the conclusion that the 159-kDa cross-link band is a dimer of domain III-V. That we observe only cross-link dimers and not significant levels of higher order proteins likely reflects that the single lysine in domain III is not symmetrically distributed within either the native DnaX tetramer or cross-linked species after distortions induced by the initial cross-link.

We observed that both γ and τ, which are each homotetramers in solution (29), produced principal BS3-dependent cross-linking products that migrate as 153 kDa (compare *lanes 5* and *6*) and 219 kDa (compare *lanes 7* and *8*), respectively. These bands probably represent dimeric species that migrated larger than their predicted size (95 and 142 kDa, respectively) for reasons explained in the preceding paragraph. Therefore, just like domain III-V, cross-linking of τ and γ produced one high molecular weight cross-link band as the only significant cross-linked species, suggesting that the DnaX tetramer is not symmetrical but perhaps a dimer of dimers. This would permit cross-linking between two protomers within the dimer but not cross-linking between associated dimers (or vice versa). Domain III, absent in the domain I-II protein, is the only domain in common to domain III-V, γ, and τ implying that BS3 cross-linking is domain III-dependent. We have demonstrated previously that domain IV-V is monomeric and does not form higher ordered complexes either with itself or full-length DnaX (26).

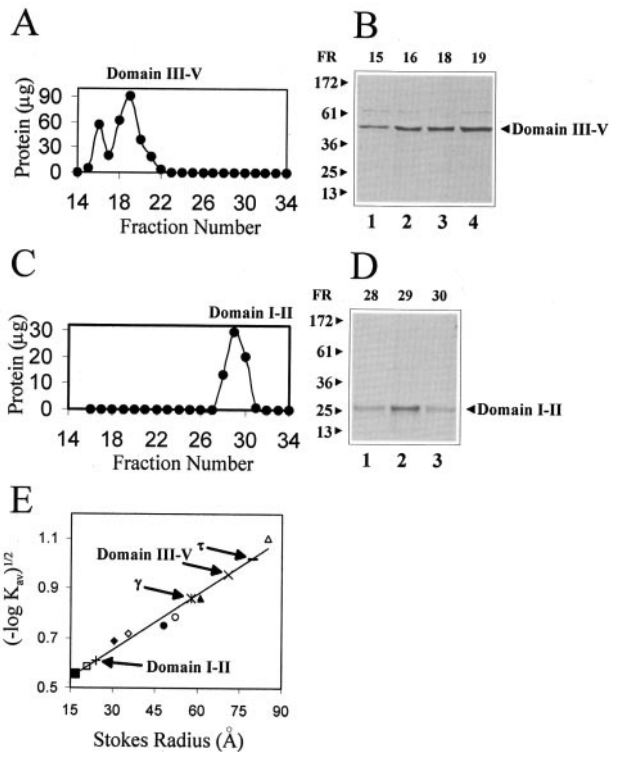

FIG. 3. **Domain III oligomerizes DnaX.** Domain I-II, domain III-V, and protein standards were gel-filtered on a 24-ml Superose 12 FPLC column as described under "Experimental Procedures." *A*, elution profile of domain III-V. *B*, samples of peak fractions containing domain III-V were boiled in SDS sample buffer and subjected to 10% SDS-PAGE analysis. Gels were stained with Coomassie Brilliant Blue R-250. *C*, elution profile of domain I-II. *D*, Coomassie-stained SDS-PAGE gel of domain I-II peak fractions. *E*, a plot to determine the Stokes radius of domain III-V (*X*) and domain I-II (+). Standards were thyroglobulin (△), ferritin (▲), catalase (○), aldolase (●), albumin (◇), ovalbumin (◆), chymotrypsinogen A (□), ribonuclease A ■), τ (—), and γ (*). The void volume (7.5 ml) was determined using blue dextran.

Because we observed high molecular weight cross-link bands only in DnaX proteins that contain domain III suggests that BS3 cross-links arise from a domain III-domain III self-interaction within the same DnaX homooligomer.

Domain III Oligomerizes DnaX—The ability of domain III-containing DnaX proteins to produce high molecular weight cross-link bands suggests that domain III-V forms an oligomer, and domain I-II is monomeric. To test this hypothesis further, we gel-filtered domain I-II and domain III-V in addition to protein standards on a 24-ml Superose 12 FPLC column. We found that domain III-V eluted in fractions 16–21 corresponding to an elution volume of 9.5 ml (Fig. 3, *A* and *B*). Fraction 16 contained aggregated domain III-V protein running near the void volume just like we often observe for a fraction of full-length recombinant DnaX during the purification. Domain I-II eluted as a single peak in fractions 28–30 corresponding to an elution volume of 14.5 ml (Fig. 3, *C* and *D*). A plot of elution position of domain I-II and domain III-V relative to standards (Fig. 3*E*) yielded Stokes radii of 24 and 70 Å for domain I-II and domain III-V, respectively. Gel filtration indicates that domain I-II elutes at a volume consistent with its monomeric molecular mass of 28.1 kDa, while domain III-V appears much larger than its monomeric molecular mass of 49.9 kDa. Using the Stokes radii of domain I-II and domain III-V alone, without correction for asymmetry, we obtain predicted globular molecular masses of 30.1 kDa for domain I-II and 490 kDa for domain III-V.

As a control we gel filtered γ and τ and determined their Stokes radius to be 57 and 79 Å, respectively. These radii are

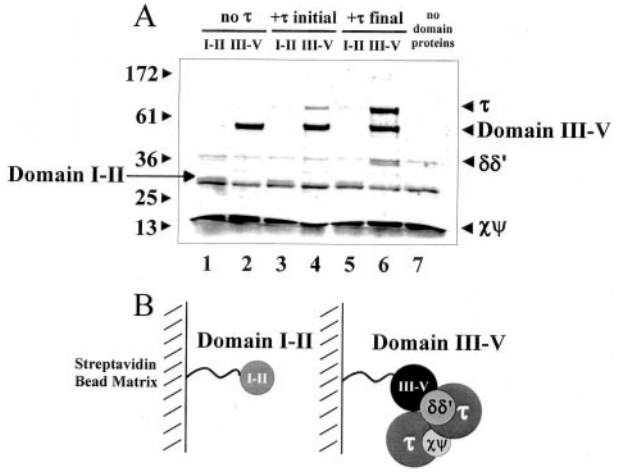

FIG. 4. **Domain III-V assembles into functional mixed DnaX complexes.** *A*, domain III-V and domain I-II was assayed for their ability to form mixed DnaX complexes. Complexes formed by incubating the domain proteins with non-biotin-tagged τ, followed by the addition of $\delta\delta'\chi\psi$, were purified using streptavidin-coated agarose beads as described under "Experimental Procedures." *Lanes 1* and *2*, domain I-II and domain III-V only; *lanes 3* and *4*, zero time point for DnaX complex assembly; *lanes 5* and *6*, DnaX complex assembly after 2-h incubation; and *lane 7*, no DnaX proteins present in DnaX complex assembly. *B*, domain III-V recruits wild-type τ and the auxiliary proteins into a mixed DnaX complex. Domain I-II, which lacks domain III, cannot form a DnaX complex.

similar to the reported values of 67 Å for γ and 80 Å for τ (40). Using the measured Stokes radii of γ and τ we obtain predicted molecular masses of 312 and 611 kDa, respectively. These predicted values are substantially larger than the calculated values expected for γ and τ tetramers (190 and 284 kDa) if the proteins behaved as ideal globular proteins. This aberrant behavior observed with γ and τ was also observed with the oligomeric domain III-V protein. To determine a molecular mass for domain III-V accounting for the asymmetry of DnaX oligomers, a standard graph was prepared correlating the apparent and the calculated tetrameric molecular masses of τ and γ. Using the experimentally determined domain III-V uncorrected molecular mass of 490 kDa, a value of 245 kDa was determined for the domain III-V oligomer from the standard graph. Taken together, these data indicate that domain I-II is monomeric, lacking the oligomerization domain, whereas domain III-V does contain the protein interaction surface (domain III) required for DnaX self-association.

Domain III Facilitates Mixed DnaX Complex Assembly—In the absence of the other holoenzyme subunits, DnaX exists as a tetramer. We have demonstrated that the association of the auxiliary subunits δ, δ', χ, and ψ with domain III of $DnaX_4$ results in a DnaX complex with an overall stoichiometry of $DnaX_3\delta\delta'\chi\psi$ (20, 29). After having identified domain III as the sequence necessary to facilitate DnaX oligomerization, we determined whether domain III-V could assemble into a mixed DnaX complex containing τ and the auxiliary subunits δ, δ', χ, and ψ. We examined whether domain III could bind τ along with δ, δ', and $\chi\psi$ forming a mixed DnaX complex. We have previously demonstrated that complexes containing biotin-tagged DnaX can be purified on streptavidin-coated agarose beads and then eluted from the beads and analyzed by Coomassie-stained SDS-PAGE gels (36). We found that individually, the biotin-tagged domain I-II and domain III-V proteins were capable of binding streptavidin-coated agarose beads (Fig. 4*A*, *lanes 1* and *2*). Domain I-II protein (*arrow*), migrating slightly larger than a contaminant protein band from the streptavidin beads, is much less intense than the domain III-V protein (*arrow*), although equimolar amounts of each were applied to the beads. This result is consistent with domain I-II binding as a monomer but domain III-V as an oligomer. The fact that only a portion of expressed fusion proteins are biotinylated magnifies the difference in domain I-II *versus* domain III-V binding to beads. From studies with the intact biotin-tagged τ fusion protein, we know that only 10–30% of the total protein applied to the streptavidin beads actually binds, and it can be calculated, assuming a binomial distribution of biotinylated τ, that only 3–8% of τ fusion protein monomers are biotinylated (36). Most of the tetramers that bind to the beads therefore contain only one biotinylated monomer. If domain III-V binds to the beads as a tetramer with only one biotin per tetramer, and domain I-II binds as a monomer, the former would bind 7-fold more protein than the latter if differences in monomer molecular masses are also considered. A 6-fold difference was measured by scanning the gel (Fig. 4*A*, *lanes 1* and *2*) with a laser densitometer.

Domain I-II or domain III-V was incubated with wild-type τ, lacking a biotin tag, to allow τ protomers to interact with protomers of tagged domain proteins. It has been demonstrated that the addition of the auxiliary subunits δ, δ', χ, and ψ prevents the incorporation of a DnaX protomer into a DnaX complex (36, 41). We incubated a mixture of τ and the auxiliary subunits, which form a τ-only DnaX complex, with domain I-II or domain III-V to establish a "zero" time point (Fig. 4*A*, *lanes 3* and *4*). No τ is seen associated with domain I-II, and only a small amount of τ is bound to domain III-V. However, when domain I-II or domain III-V was incubated with τ for 2 h before the reaction was quenched by the addition of δ, δ', χ and ψ, a large amount of τ is seen associated with the domain III-V protein but none with the domain I-II protein (Fig. 4*A*, *lanes 5* and *6*). This demonstrates that domain I-II could not recruit τ and the auxiliary subunits onto the beads, while domain III-V, because it contained domain III, was able to form a mixed DnaX complex containing τ, δ, δ', χ, and ψ. In a control experiment containing τ, δ, δ', χ, and ψ,but no tagged domain protein, no τ was seen associated with the streptavidin beads due to nonspecific interactions. (Fig. 4*A*, *lane 7*). We conclude domain III-V is capable of forming a DnaX complex with τ and the auxiliary subunits (Fig. 4*B*).

DISCUSSION

The DnaX complex from the DNA polymerase III holoenzyme is a multiprotein subunit complex that assembles the β processivity factor onto DNA in an ATP-dependent reaction. In this study, we identified the DnaX oligomerization domain by examining the properties of truncated τ proteins lacking specific domains. Our results indicate that domain III (amino acid residues 222–382) shared by γ and τ binds to domain III of other DnaX protomers, leading to oligomerization.

BS3, which chemically cross-links primary amines, has been used extensively as an assay for protein-protein interactions, not only between different proteins, but also between protomers within homooligomeric complexes (6, 37–39). We employed BS3 cross-linking to determine whether domain III functions as the oligomerization domain in DnaX. As a positive control, we cross-linked tetrameric DnaX proteins, which contain domain III (γ and τ) and a protein that lacks domain III (domain I-II). Previously we have demonstrated that the portion of DnaX present in τ and absent in γ (domain IV-V) is monomeric and does not form oligomers either with itself or with full-length DnaX (29). We observed that BS3 cross-linking of domain III-V, τ, and γ, but not domain I-II, resulted in the appearance of high molecular mass cross-link bands. Specifically, cross-linking results with domain III-V mirrored those of the positive control reactions with τ and γ. When domain III was absent (negative control) in the DnaX protein (domain

I-II), no high molecular mass cross-link bands are observed. Because BS3 cross-linking was only observed in DnaX proteins containing domain III, and that both γ and τ have been previously demonstrated to be oligomeric, implies that domain III-V is also oligomeric.

In addition to the cross-linking results, the Stokes radii measured for domain III-V (70 Å) and domain I-II (24 Å) demonstrate different oligomerization states for domain III-V and domain I-II. Domain I-II behaved as a monomeric protein in gel filtration, while domain III-V eluted at a volume consistent with it being oligomeric. Just like in the purification of native DnaX, we observed some aggregated, presumably incompletely folded, material eluting in the excluded volume. The majority of domain III-V eluted in the included volume in a defined peak and at a molecular mass consistent with it being an asymmetrically shaped tetramer just like τ and γ. Oligomers of DnaX proteins are asymmetric in shape as the Stokes radii of domain III-V, γ, and τ predict molecular mass values for the proteins substantially larger than values expected for tetrameric forms (5). Using the experimentally determined domain III-V molecular mass of 490 kDa, a value of 245 kDa was estimated for the domain III-V oligomer from a standard graph. The estimated molecular mass of domain III-V suggests that it exists either as a tetramer or pentamer in solution. This value is an approximation and may reflect domain III-V's further asymmetry over γ and τ due to its long unstructured biotin/hexahistidine tag absent in the native DnaX standards. Nevertheless, whether domain III-V is a tetramer or pentamer does not change the overall conclusion that DnaX proteins containing domain III are oligomeric, while those lacking domain III are monomeric. The concentration of domain III-V and domain I-II in the gel filtration column load and peak fraction was 35 and 3.6 μM (as monomer) for domain III-V and 22 and 2.1 μM (as monomer) for domain I-II. The K_D for the γ and τ monomer-tetramer equilibrium is 170 nM (29). Assuming that a single domain contains all the protein sequence required for DnaX oligomerization, either domain I-II or domain III-V would be expected to be able to form a DnaX oligomer under the gel filtration experimental conditions if it contained the self-association domain. This infers that the inability of domain I-II to oligomerize was not due to dilution on the gel filtration column but because it lacked domain III.

Domain III-V recruited full-length τ into heteromeric DnaX complexes containing δ, δ', χ, and ψ. Using a streptavidin-coated bead procedure, we found that the presence of domain III was sufficient to form a DnaX complex, which contained domain III-V, τ, δ, δ', χ, and ψ (Fig. 4B). Domain I-II, which lacks domain III, was unable to recruit τ, δ, δ', χ, and ψ onto the beads. We have demonstrated previously that domain IV-V present in τ is unable to form oligomers with itself and cannot bind the auxiliary subunits δ, δ', χ, and ψ (26). This demonstrates that domain III not only binds the auxiliary subunits (20) but also functions as the DnaX oligomerization domain recruiting τ to the beads through a direct domain III-domain III interaction.

Like clamp-loading complexes from eukaryotes, subunit components within the DnaX complex share both a sequence and possible structural similarity with each other. The auxiliary subunit δ' shares a high sequence similarity to the N-terminal domain I-III of DnaX but cannot form higher homo-oligomeric complexes (27, 28).[2] δ' does bind to DnaX and plays a key role in the $DnaX_4$ to $DnaX_3$ transition observed during DnaX complex formation (29). We have proposed that this mechanism involves the replacement of one homologous protein (one DnaX protomer) with another (δ') during complex formation. Perhaps it is because DnaX oligomerizes through domain III that the binding of δ' to domain III affects the oligomerization state of τ and γ. When δ' binds to domain III, it may replace a DnaX protomer by substituting a DnaX-DnaX interaction with a DnaX-δ' contact.

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[2] M. Song, H. G. Dallmann, and C. S. McHenry, unpublished data.