Assembly of DNA Polymerase III Holoenzyme

CO-ASSEMBLY OF γ AND τ IS INHIBITED BY DnaX COMPLEX ACCESSORY PROTEINS BUT STIMULATED BY DNA POLYMERASE III CORE

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Although the two alternative Escherichia coli dnaX gene products, τ and γ, are found co-assembled in purified DNA polymerase III holoenzyme, the pathway of assembly is not well understood. When the 10 subunits of holoenzyme are simultaneously mixed, they rapidly form a nine-subunit assembly containing τ but not γ. We developed a new assay based on the binding of complexes containing biotin-tagged τ to streptavidin-coated agarose beads to investigate the effects of various DNA polymerase III holoenzyme subunits on the kinetics of co-assembly of γ and τ into the same complex. Auxiliary proteins in combination with δ̂ almost completely blocked co-assembly, whereas ψ alone slowed the association only moderately compared with the interaction of τ with γ alone. In contrast, DNA polymerase III core, in the absence of δ̂ and ψ, accelerated the co-assembly of τ and γ, suggesting a role for DNA polymerase III' [(τγ)2(pol III core)2] in the assembly pathway of holoenzyme.

The Escherichia coli chromosome is replicated by the DNA polymerase III holoenzyme,1 which contains three functional subassemblies: pol III core, the δ̂ sliding clamp processivity factor, and the DnaX complex. The pol III core contains the α, ε, and θ subunits and provides the polymerase function. The multiprotein DnaX complex recognizes the primer terminus, loads β onto DNA in an ATP-dependent manner, and functions as a communications and organizational node for the various replication and primosomal proteins at the replication fork (1–9).

The DnaX complex contains the ATPases τ and γ, which are the alternative frameshift products of the dnaX gene, plus the auxiliary subunits δ̂, δ̂', χ, and ψ. The τ subunit, but not the shorter translation product γ, dimerizes the pol III core through interactions between structural domain V of τ and the α subunit to coordinate leading and lagging strand synthesis (6, 10–12). There is also a τ-mediated interaction between holoenzyme and DnaB that is essential for coupling the replication and the primosomal apparatus at the replication fork (4, 7, 9, 12). In the elongation complex, τ protects β from removal by exogenous γ complex, increasing the processivity of the replication (12). The τ subunit also is a bridge between α and a χ single-stranded DNA-binding protein interaction, strengthening the holoenzyme interactions with the protein that coats the lagging strand at the replication fork (13, 14).

Within the DnaX complex, δ̂ and ψ bind directly to γ; δ̂ binds δ̂', and χ binds ψ (5, 15). The DnaX-δ̂ and DnaX-ψ interactions occur through structural domain III, which is common to both τ and γ (8). It is also known that δ̂ and δ̂' form a 1:1 complex and together with DnaX load β onto primed templates (16, 17). The χ and ψ subunits also form a 1:1 complex and increase the affinity of DnaX for δ̂ and δ̂' such that a functional DnaX complex can be assembled at physiological subunit concentrations (8, 13, 15, 18).

Various forms of the clamp-loading complex have been characterized including a γ complex [(γδ̂δ̂'χψ), a τ complex (τδ̂δ̂'χψ), and two different τγ mixed DnaX complexes (τγγδ̂δ̂'χψ and τγγδ̂δ̂'χψ) (15, 17, 19–21)]. A novel assembly mechanism for the DnaX complex has been discovered recently: free DnaX is a tetramer in equilibrium with a free monomer (Kp = 170 nM), but the DnaXγ stoichiometry is altered upon δ̂ association, leading to the formation of a DnaXγδ̂γ′ complex (21).

Both τ and γ are found co-assembled in purified holoenzyme and in pol III*, a subassembly of holoenzyme that lacks only β (22, 23), but the assembly mechanism is not well understood. When the 10 subunits of holoenzyme are mixed simultaneously, they rapidly form a nine-subunit assembly containing τ but not γ (15). An alternative pathway through pol III', an isolable subassembly comprised of τγ(pol III core)2 (10), was also investigated, but the γ complex and pol III' did not associate upon mixing (17). If the entire complement of DnaX proteins is overexpressed from a single operon, τγ mixed DnaX complexes are formed and can be purified by SP-Sepharose chromatography (21). Also, two in vitro protocols have been developed that produce a pol III* that contains both τ and γ in the same complex (17).

In view of the important roles for the DnaX complex in replication and its unusual mechanism of assembly, we investigated the effects of various accessory proteins on the time course of the co-assembly of τ and γ. We hoped to discover the factors required for the assembly of τ and γ into the same complex and to eventually dissect the steps in the assembly pathway. In addition, efficient in vitro assembly of a proper mixed complex will be useful in future studies on the roles of specific proteins and their interactions. We have determined that τγ association proceeds slowly and the presence of the DnaX complex auxiliary proteins impedes this association. We looked at the assembly of τ and γ into pol III* and discovered that pol III core, in the absence of DnaX core accessory proteins, stimulates co-assembly, suggesting that the holoen-
zyme assembly pathway proceeds through the initial formation of pol III'.

**EXPERIMENTAL PROCEDURES**

**Buffers**—The buffers used were: buffer SP (50 mM Tris (pH 7.5), 10% (v/v) glycerol, 5 mM DTT); buffer 25TS (25 mM Tris (pH 7.5), 5% (v/v) glycerol, 5 mM DTT); buffer G (20 mM Tris (pH 7.5), 25 mM NaCl, 0.1 mM EDTA, 20% (v/v) glycerol, 5 mM DTT); buffer SW (20 mM Tris (pH 7.5), 200 mM NaCl, 0.02% Nonidet P-40, 20% (v/v) glycerol, 5 mM DTT); and buffer T2 (20 mM Tris (pH 7.5), 20% (v/v) glycerol, 5 mM DTT).

**Protein Purification**—C(O) is the subunit with a C-terminal fusion peptide that includes a short 13-amino acid biotinylated cloning site. A hexahistidine sequence, and a thrombin cleavage site. The activity of C(O) includes a short 13-amino acid biotinylation sequence, a 200 mM NaCl, 0.02% Nonidet P-40, 20% (w/v) glycerol, 5 mM DTT); buffer SW (20 mM Tris (pH 7.5), 200 mM NaCl, 0.02% Nonidet P-40, 20% (v/v) glycerol, 5 mM DTT); and buffer T2 (20 mM Tris (pH 7.5), 20% (v/v) glycerol, 5 mM DTT).

**Pol III core** if appropriate) but not C(O)III core if appropriate) but not γ. Background stain intensity at the position expected for the γ subunit was measured as a percentage of the C(O)III core stain intensity. This value, which was used as the background stain intensity for the C(O)III core stain intensity. The amount of C(O)III core was determined by measuring the intensity of the C(O)III core signal and was subtracted (as a percentage of the C(O)III core stain intensity) for each of the time points. 3) A zero-point control was accomplished by adding, on ice, C(O)III core to a preincubated mixture of γ, δ, δ', and χ, which comprised the quench. Higher levels than expected of γ associated with C(O)III core were seen in this control (γ/molar ratios ranging from 0.08 to 0.18). This did not indicate that the quench was ineffective, because the γ/molar ratios in the presence of δδ' ψ χ also gave similar results. It is possible that the γ seen associated with C(O)III core in this control is a consequence of the streptavidin bead procedure or caused by aggregation of C(O)III core and γ proteins. The γ'/molar ratios of the zero point control was subtracted from all time points.

**SP Chromatography**—For experiments investigating the effect of SP-Sepharose on C(O)III core to C(O)III core, the wash was made with an equilibration buffer containing 30% methanol and 5% acetic acid. The gel was destained overnight in at least two changes of 20% methanol and 5% acetic acid.

**RESULTS**

**Assay to Study Kinetics of Association of γ and γ'**—The two DnaX proteins, γ and γ', associated with other subunits of holoenzyme, exist as tetraters in equilibrium with monomers. If purified γ and γ' are mixed they can associate to form a mixed tetramer, a reaction that is blocked by the presence of the DnaX complex auxiliary proteins, δ, δ', χ, and γ (17). Here, we refer to the overall assembly of γ and γ' as an exchange of DnaX protomers in complexes initially homomeric in DnaX. To study the parameters affecting these exchange reactions, we developed an assay that takes advantage of a biotin tag near the C terminus of C(O)III core. γ' was allowed to exchange into complexes with C(O)III core under varying experimental conditions, the exchange reaction was quenched at various time points by addition of the C(O)III core if appropriate) but not γ. Background stain intensity at the position expected for the γ subunit was measured as a percentage of the C(O)III core stain intensity. This value, which was used as the background stain intensity for the C(O)III core stain intensity. The amount of C(O)III core was determined by measuring the intensity of the C(O)III core signal and was subtracted (as a percentage of the C(O)III core stain intensity) for each of the time points. 3) A zero-point control was accomplished by adding, on ice, C(O)III core to a preincubated mixture of γ, δ, δ', and χ, which comprised the quench. Higher levels than expected of γ associated with C(O)III core were seen in this control (γ/molar ratios ranging from 0.08 to 0.18). This did not indicate that the quench was ineffective, because the γ/molar ratios in the presence of δδ' ψ χ also gave similar results. It is possible that the γ seen associated with C(O)III core in this control is a consequence of the streptavidin bead procedure or caused by aggregation of C(O)III core and γ proteins. The γ'/molar ratios of the zero point control was subtracted from all time points.
forming a complete DnaX complex (DnaX3/\delta\psi), and complexes containing \gamma were purified away by the binding of biotin-tagged C(O) to streptavidin beads. The purified complexes were electrophoresed on SDS-polyacrylamide gels, and the molar ratios were determined.

An example of the procedure, the \gamma exchange in the presence of \delta (Fig. 1), shows that a \gamma mixed DnaX complex did form, and the relative amount of \gamma increased with time. However, when \psi as well as \delta were present, the amount of \gamma did not increase with time (Fig. 1B). Thus, the association of C(O)\tau and \gamma was blocked in the presence of \delta plus \psi, which is consistent with an earlier study showing that \delta\psi prevented co-assem-
was eluted in 0.25-ml fractions with a 20-ml 20 to 300 mM NaCl gradient run at either 0.5 ml/min (profile labeled fast) or 0.02 ml/min (profiles labeled slow). The individual profiles were aligned by the salt gradients measured for each run. The proteins present in the load of each column are indicated on the left, and Coomassie Blue-stained SDS 10% polyacrylamide gels of peak fractions, labeled on each profile, are shown on the right. The proteins δ and δ' co-migrate on the gels.

In each series of experiments a control, in which the bead wash procedure removed nonbiotinylated proteins that were not associated with C(O)γ was performed (untagged γ, Fig. 1B). Another control, lacking only γ, was used to subtract any background signal in the gel (no γ lane, Fig. 1B) caused by for example minor contaminants migrating at the γ position. In the zero point control (Zero Pt lane, Fig. 1B), C(O)γ was added to a reaction at 0 °C that was quenched already because it contained γ preincubated with δδ'χψ. The γ/τ molar ratio for zero-point control was subtracted from all of the experimental time points.

Subunits That Prevent the Association of τ and γ—When C(O)τ was added to a preformed complex of γδδ'χψ maintained at 15 °C, there was no increase in the γ/τ ratio over time (Fig. 2A); δδ'χψ blocked the association of C(O)τ and γ, confirming an earlier study (17). After 120 min the γ/τ ratio was zero after the background control, and zero-point corrections were applied. In contrast, when C(O)τ and γ were allowed to exchange in the absence of accessory proteins, the average γ/τ ratio was 1.0 after 120 min. This result demonstrates the effectiveness of the quench used in the assay. Two other combinations of subunits, δδ' and δ'χψ, were nearly as effective in suppressing the association of C(O)τ and γ with γ/τ ratios of 0.17 and 0.02, respectively, after 120 min. All the accessory protein combinations that exhibited the maximal kinetic inhibition contain δ' (Fig. 2A).

Subunits That Slow the Association of τ and γ—Other combinations of subunits, when allowed to form complexes with γ, slowed but did not abrogate the association of C(O)τ and γ into the same complex (Fig. 2B). The curves for the C(O)τ plus γψ or γδ' complexes were overlapping and revealed slightly slower kinetics than the C(O)τ plus γ only curve. After 80 min the average γ/τ ratio was 1.0 with no added accessory proteins, 0.7 in the presence of χψ, and 0.66 in the presence of δ'. We did not observe a significant effect on the γτ association kinetics when only δ was added to the reaction (data not shown). Similarly, the curve for the association of C(O)τ and γ in the presence of χψ was not altered when δ was also included (data not shown). Thus, any accessory protein that binds directly to DnaX in the complex retards rather than accelerates the association τ and γ. The magnitude of the inhibition varies for the different combinations of proteins tested.

Pol III Core Accelerates the Association of τ and γ—The effect of another τ-binding protein, pol III core, on the assembly of τ and γ into pol III* was also examined. Because pol III core binds to τ but not to γ, we first incubated τ with a 2-fold molar excess of pol III core for 10 min before adding γ to initiate the exchange reaction. The reactions were quenched at various times by the addition of δδ'χψ. Pol III core accelerated the γτ exchange reaction approximately 3-fold compared with the reaction with only τ and γ present (Fig. 2C). After only 2 min, the γ/τ ratio was increased from 0.15 to 0.40. This rate increase is in marked contrast to the inhibitory effects of the other τ-binding protein. The actual measured rate increase depends on
solution conditions and possibly the presence of the C-terminal tag on C(O)τ. It is known that the $K_p$ for the α-C(O)τ association is in the nM range compared with a pM range for the interaction of α with the N-terminal fusion τ (6), but because our experiments were conducted in the μM range, it is unlikely that the decreased α-τ affinity caused by the C-terminal tag affected the results.

It should be noted that we did not observe γ associated with pol III in the absence of added δδ' and χψ (data not shown). However, δδ' and χψ were not responsible for stimulating the association kinetics, only for trapping an intermediate, because if δ, δ', χ, and ψ were present along with pol III' when γ and τ were mixed, no γ was found associated with τ. This indicates that γ can interact with pol III' in a time-dependent reaction, but the association is weak and does not survive the streptavidin bead-washing procedure without forming a stable DnaX complex.

**SP-Sepharose Chromatography of the γτ Complex—**It is clear from the streptavidin bead assay with C(O)τ and from previous work (17) that τ and γ by themselves can associate in vitro to form a heterologous DnaX oligomer. Yet it has also been demonstrated that a mixture of τ and γ, even if obtained by co-expression from a plasmid, elute from S-Sepharose as separate τ and γ peaks with no evidence of a mixed γτ complex (25). This enigma was investigated in a series of SP-Sepharose chromatography experiments (Fig. 3). To form a γτ mixed complex, τ was allowed to associate with a 2-fold molar excess of γ at 15 °C for 1.5 h, which is enough time to form an equilibrated mixed complex based on our streptavidin bead experiments. The resulting mixture was then chromatographed on a 1-ml SP-Sepharose column that was developed with a 20-ml 20–300 mM NaCl gradient run slowly (0.02 ml/min). The two DnaX proteins eluted separately as γ and τ peaks at salt concentrations equivalent to ~100 and 210 mM NaCl, respectively. There was no evidence of any mixed γτ oligomers confirming our earlier observation (25). However, when the SP-Sepharose column gradient was developed rapidly (0.5 ml/min), in addition to the γ peak several overlapping peaks comprising γτ mixed complexes were observed eluting between 155 and 190 mM NaCl. The γτ ratio in the eluted complex increased with increasing salt concentration. Although the mixed complex species were not purified to homogeneity for characterization, we expect that there were three species present corresponding to γτγτ, τγτγ, and τγτγ. The SP-Sepharose method therefore promotes the dissociation of γτ mixed oligomers, a process that was completed if the proteins were eluted at a slow flow rate but not at the faster flow rate.

Can accessory DnaX complex subunits stabilize the γτ mixed complex subassemblies and prevent dissociation on SP-Sepharose? After the association of τ and γ at 15 °C for 1.5 h, three combinations (δδ'χψ, δδ', or χψ) of the auxiliary proteins in separate experiments were added to the γτ mixture, and the resulting subassemblies were chromatographed at the slow rate of 0.02 ml/min (Fig. 3). In all three experiments, we observed subassemblies containing both τ and γ eluting between 170 and 210 mM NaCl. Therefore, δδ'χψ, δδ', or χψ can stabilize γτ mixed oligomers against dissociation on the column. However, δδ' was more effective than χψ in stabilizing mixed γτ complexes (compare the bottom panel of Fig. 3 with the two panels above it).

**DISCUSSION**

We have shown that various combinations of the auxiliary DnaX complex proteins, δ, δ', χ, and ψ, slow the co-assembly of τ and γ into the same complex; τ and γ exchange into the same complexes faster in the absence of these proteins. This result implies that if the holoenzyme assembly pathway is through the DnaX complex, the formation of a γτ mixed oligomer occurs before any other auxiliary protein enters the complex. Because τ and γ are translated from the same RNA, co-translational formation of a mixed DnaX oligomer before the accessory proteins become associated is likely.

Any DnaX complex accessory protein that is known to bind to DnaX slows the exchange of γ into τ-containing complexes. The accessory proteins interact with DnaX through its domain III (8), where the DnaX oligomerization domain is also located. All the auxiliary protein combinations that prevent the exchange of γ and τ into the same complex include δ'. Although δ', which binds very weakly to DnaX by itself, is an ineffective inhibitor, all agents that synergistically increase the apparent affinity of δ' for DnaX (δ and/or χψ) enhance the efficacy of δ'-mediated inhibition. We know that the binding of δδ' promotes a DnaXδδ' transition, an event that may tighten DnaX-DnaX interactions precluding exchange.

For the association of τ and γ in the absence of any other proteins, the C(O)τ and γ concentrations are 5.3 and 11 μM, respectively, and therefore, from the $K_p$ of 170 mM (21), ~7 and 4% of the two DnaX proteins, respectively, exist in a monomeric rather than a tetrameric state. The association of τ and γ in vitro could occur hypothetically via either oligomeric state.

In contrast to the DnaX accessory proteins, pol III core stimulates the co-assembly of τ and γ into the same complex; τ incubated with pol III core and then mixed with γ co-assembles faster than τ and γ incubated alone. This result suggests a pathway for the formation of pol III* in the cell via pol III (τγ) (pol III core). We know from previous work that our protocol used in the kinetic analysis, incubating C(O)τ with a 2-fold molar excess of pol III core, reconstitutes pol III* in vitro (11). A pathway via pol III* is particularly attractive, because the holoenzyme replicate almost certainly contains two τs and one γ, whereas free DnaX, unassociated with any accessory protein, is a tetramer in equilibrium with a free monomer (21). Pol III core may stimulate γτ co-assembly by dissociating the DnaX tetramer, a potential kinetic barrier in the assembly pathway (Fig. 4). The fact that pol III* mixed with γ complex is a dead end in the assembly pathway (17) is because of the stability of the γτδδ'χψ complex.

Based on our observations and knowledge of the mechanism of DnaX synthesis, we favor a model in which DnaX forms heterologous γτ oligomers by co-translational assembly from the same DnaX mRNA (Fig. 4). High local concentrations of DnaX should facilitate rapid interaction. Then, pol III is proposed to bind to τ-containing complexes, with those containing two or more τs being favored in the formation of pol III*. Monomeric γ in equilibrium with DnaX tetramers could then associate with pol III* weakly, forming a heterologous intermediate. This unstable pol III*-γ intermediate is then proposed to associate with δδ' and χψ, which stabilize DnaX-DnaX interactions and favor the formation of stable pol III* (Fig. 4).

**REFERENCES**


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