DnaX Complex of Escherichia coli DNA Polymerase III Holoenzyme

THE $\chi$-$\psi$ COMPLEX FUNCTIONS BY INCREASING THE AFFINITY OF $\tau$ AND $\gamma$ FOR $\delta$-$\delta'$ TO A PHYSIOLOGICALLY RELEVANT RANGE* 

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An artificial operon that contains tandem holC-holD genes was used to overproduce a complex of the $\chi$ and $\psi$ subunits of the DNA polymerase III holoenzyme. Normally insoluble by itself, $\psi$ forms a tight soluble complex with $\chi$. A purification procedure that yields pure, active $\chi$-$\psi$ complex in 100-mg quantities suitable for biophysical studies is reported. Sedimentation equilibrium studies demonstrate that $\chi$-$\psi$ is a 1:1 heterodimer. The presence of $\chi$-$\psi$ dramatically lowers the level of $\delta$-$\delta'$ required to reconstitute holoenzyme to levels expected in vivo. That $\chi$-$\psi$ accomplishes this by binding to $\gamma$ or $\tau$ and increasing their affinity for $\delta$-$\delta'$. The $\chi$-$\psi$ complex was demonstrated by surface plasmon resonance using a Pharmacia BIACORE™ instrument. In the absence of $\delta$-$\delta'$, $\chi$-$\psi$ binds to either the $\gamma$ or $\tau$ DnaX protein with $K_d = 2 \text{nm}$. The $\chi$-$\psi$ complex setting apparatus contains either the $\gamma$ or $\tau$ auxiliary subunit that confers the special properties expected of a replicative polymerase (for reviews, see McHenry (1991) and Kuriyama and O'Donnell (1993)). These include high processivity and the ability to communicate with primosomal proteins at the replication fork to permit coordinated replication (Wu et al., 1992a, 1992b). The holoenzyme auxiliary subunits can be divided into two subassemblies: 1) $\beta$ forms a sliding clamp that apparently encircles DNA (Kong et al., 1992) and tethers the $\eta$ core to the template by protein-protein interactions (LaDuca et al., 1986; Stukenberg et al., 1991); and 2) the DnaX complex sets the sliding clamp onto the template-primer (Wickner, 1976).

The DnaX clamp-setting apparatus contains either the $\tau$ or $\gamma$ DnaX gene product complexed to $\delta$-$\delta'$ and $\chi$-$\psi$ (McHenry et al., 1986; Maki and Kornberg, 1988, Xiao et al., 1993b; Dallmann and McHenry, 1995). The $\tau$ and $\gamma$ subunits are ATPases within the clamp-loading assembly (Lee and Walker, 1987; Hawker and McHenry, 1987; O'Donnell et al., 1993). Presumably, these subunits function to couple the energy achieved from ATP hydrolysis to the assembly of the $\beta$ sliding clamp. The $\tau$ subunit also functions to dimerize pol III by direct contact with the $\alpha$ subunit (McHenry, 1982; Studwell-Vaughan and O'Donnell, 1991). The ATPase activities of $\tau$ and $\gamma$ are stimulated by the presence of $\delta$-$\delta'$ or $\chi$-$\psi$ (Onrust and O'Donnell, 1993; Xiao et al., 1993a), suggesting direct binding of one of the subunits. Gel filtration of mixed subunits shows that $\delta$ and $\delta'$ bind weakly to $\tau$ and $\gamma$ to form a complex (Onrust and O'Donnell, 1993). In a minimal holoenzyme assembly, a strong requirement is observed for both the $\delta$ and $\delta'$ subunits (Onrust and O'Donnell, 1993).

The $\chi$ and $\psi$ subunits have not been assigned a clear function. They were initially identified by their association with purified $\gamma$ complex (McHenry et al., 1986; Maki and Kornberg, 1988), and it was not clear until they had been partially sequenced and their structural genes cloned that they were distinct proteins instead of proteolytic products of $\delta$ or $\delta'$. Xiao et al., 1993a, Carter et al., 1993b). No requirement has been observed for $\chi$-$\psi$ other than a modest stimulation of holoenzyme reconstituted with the $\gamma$ DnaX protein in the presence of elevated levels of salt (Xiao et al., 1993b). It has been shown that $\chi$ and $\psi$ form a 1:1 complex (Xiao et al., 1993b). Gel filtration studies indicated that $\chi$-$\psi$ forms a complex with $\tau$ or $\gamma$ in solution and that $\psi$ bridges the interaction of $\chi$ with $\gamma$ (Xiao et al., 1993b).

The insolvibility of the $\psi$ subunit and its tendency to aggregate has limited its utility in physical and functional studies (Xiao et al., 1992, 1993). $\psi$ required 6 M urea for all purification steps, and the resulting protein was inactive and aggregated when urea was removed. The resulting denatured purified $\psi$ was useful only if rapidly gel-filtered to remove urea immediately before conducting an experiment or if diluted to 0.5 M urea, clearly a complication for kinetic and biophysical experiments. Presumably as a result of the need to refold, assembly reactions proceeded slowly, typically requiring 30 min (Xiao et al., 1993a).

The discovery, cloning, overexpression, and purification of each subunit of the DnaX complexes has allowed us to study their contributions to the holoenzyme replicative reaction. We now report the purification and physical and functional characterization of the $\chi$-$\psi$ complex. Exploiting an artificial operon that overproduces both $\chi$ and $\psi$, we show that these subunits assemble in vivo to form a soluble 1:1 complex that is suitable for biophysical studies. We demonstrate that the most striking contribution of $\chi$-$\psi$ to the holoenzyme reaction is its ability to bind $\tau$ or $\gamma$ and increase their affinity for $\delta$-$\delta'$ so that they can form a functional clamp-loading complex at physiological subunit concentrations.
Chi Increases the Affinity of DnaX for \( \delta' \)

EXPERIMENTAL PROCEDURES

E. coli Strains and Growth—The E. coli K-12 strain MC1061 (F-, hsd R2, mcr B1, ara D139 dae-7697 [DEL], Dlac -174, gal U, gal K, rpsL, th) containing the plasmid pMAF 310 (hol'C-, hol D'-, amp R', lac Q') (Carter, et al. 1993a) was grown in a 250-liter fermentor (New Brunswick) in F-medium + glucose and ampicillin at 37°C. Cells from a growing 20-liter culture were diluted 1:10 in 180 liters of F-medium in the 250-liter fermentor. F-medium is composed of yeast extract (14 g/liter), tryptone (18 g/liter), KH2PO4 (12 g/liter), K2HPO4 (1.2 g/liter) (pH 7.2). Glucose and ampicillin are added to 1% and 50 \( \mu \)g/ml, respectively, at the beginning of the fermentation and at the point of induction with isopropyl-\( \beta \)-D-thiogalactoside (1 \( \mu \)M final concentration at OD600 = 1.0). Three hours after induction (OD600 = 3.1), cells were chilled and harvested by passing the fermentation broth through cooling coils en route to a Sharpless continuous flow centrifuge. The temperature of the effluent did not exceed 16°C. Cells were resuspended with an equal volume (w/v) of cold (4°C) Tris-sucrose (50 mM Tris-HCl (pH 7.5), 10% sucrose) and poured into liquid nitrogen as a stream. This procedure yielded 1280 g of cells.

Chromatographic Supports—Q-Sepharose, SP-Sepharose, and Sephacryl S100 were obtained from Pharmacia Biotech Inc.

Proteins—Purification of the \( \beta \) subunit (Johanson et al., 1986) and of the \( \tau \) and \( \gamma \) subunits (Dallmann et al., 1995) to homogeneity from overexpressing strains was carried out as previously described. SSB (1 mg/ml) and DnaG primase (4.2 units/ml) were purified from overproducing strains as described (Gries and McHenry 1989). DNA polymerase III holoenzyme (hol complex), holoenzyme (hol complex), holoenzyme (hol complex), or 50 \( \mu \)M, 5-\( \mu \)l injection, 20 mM sodium phosphate (pH 7.4); \( \gamma \), 50 \( \mu \)g/ml, 10 \( \mu \)l—(injection, 20 mM MES (pH 6.0)); \( \delta \), 300 \( \mu \)g/ml, 7-\( \mu \)l injection, 20 mM MES (pH 6.0); and \( \delta' \), 100 \( \mu \)g/ml, 30-\( \mu \)l injection, 20 mM MES (pH 6.0). Unreacted N-hydroxysuccinimide ester groups were pulled with a 30-\( \mu \)l injection of 1 mM ethanamine-HCl (pH 8.0). Under these conditions, typically 7000 response units (RU) of \( \gamma \), 3000 RU of \( \delta \), and 1500 RU of \( \delta' \) were immobilized. Protein-protein interaction studies were carried out in buffer HKGM at a flow rate of 5 \( \mu \)l/min at 20°C.

Analytical Ultracentrifugation—Sedimentation velocity and sedimentation equilibrium experiments were performed using a Beckman model XL-A analytical ultracentrifuge. All sedimentation experiments were performed at 4°C using a Beckman four-hole An-D6TI rotor and were carried out in 1.2-cm path length double-sector cells with quartz windows. The protein absorbance was monitored at 230 nm and 280 nm. \( \chi \) complex (fraction V) was dialyzed overnight against 3 liters of 5% glyceral, 100 mM NaCl, 2 mM DTT, 20 mM Tris (pH 7.5), and 1 mM EDTA to a final concentration of 57 \( \mu \)M. The sample contained \( \chi \) complex (4 RU/liter, 1000 RU of \( \gamma \), and 500 RU of \( \delta' \) were immobilized. Protein-protein interaction studies were carried out in buffer HKGM at a flow rate of 5 \( \mu \)l/min at 20°C.

RESULTS

In preliminary studies, we found that the \( \psi \) subunit, when overproduced by itself, formed aggregates that require denaturing conditions for solubilization. O’Donnell and colleagues published a similar observation and developed a purification for \( \psi \) that started with denatured material (Xiao et al., 1993a). They found that assays using \( \psi \) required extensive incubations, purification, and assembly of \( \psi \) into complexes. We found that \( \psi \) when overproduced from an artifical operon with \( \chi \), forms a soluble, monodisperse complex with \( \chi \) in vivo. We exploited the availability of this artificial operon (Carter et al., 1993a) to generate a \( \chi \)-\( \psi \) complex that could be purified intact without a requirement for denaturation and refolding.

Overproduction and Purification of \( \chi \)-\( \psi \)

Preparation of Cell Lysate and Ammonium Sulfate Precipitation—All operations used in the purification of \( \chi \)-\( \psi \) were performed at 0–4°C. The lysate (fraction I) was prepared from 150 g of cells (300 g of cell paste) as described by Cull and McHenry (1995)) with the following exceptions: 0.258 g of ammonium sulfate for each milliliter (45% saturation) was added to the resulting supernatant, and precipitant (fraction II) was collected by centrifugation at 22,000 \( \times \) g for 60 min. Densitometry of Coomassie-stained SDS-polyacrylamide gels indicated \( \chi \) and \( \psi \) constitute 9% and 6% of the total cellular protein (data not shown) and 7 and 4% of the total soluble protein in fraction I, respectively (Fig. 1, lane 1).

Q-Sepharose Chromatography—Fraction II (2310 mg) was dissolved in 60 ml of buffer Q and dialyzed against a total of 3 liters of buffer Q to an ionic equivalent of 20 mM NaCl (buffer Q + 20 mM NaCl). This material was loaded onto a Q-Sepharose column (750 ml; 5.8 cm x 30 cm) equilibrated with buffer Q at 0.3-column volume/h. The \( \chi \)-\( \psi \) activity eluted with an 8-column volume linear gradient from 20 to 200 mM NaCl in buffer Q at 0.5-column volume/h. Peak fractions (200 ml) were
combined and precipitated with ammonium sulfate (65% saturation) for 3 h and collected by centrifugation at 28,000 × g for 60 min.

SP-Sepharose Chromatography—Fraction III (535 mg) was dissolved in 18 ml of buffer SP and dialyzed against 3 liters of buffer SP overnight to an ionic equivalent of 200 mM NaCl. This material was loaded onto an SP-Sepharose column (350 ml; 4 cm × 28 cm) previously equilibrated with 3.5 liters of buffer SP at pH 7.5. The χψ activity was eluted with a 3-column volume linear gradient from 20 to 200 mM NaCl in buffer SP at 0.5-column volume/h. Peak fractions (158 ml) were combined and precipitated with ammonium sulfate (65% saturation) for 3 h and collected by centrifugation at 28,000 × g for 60 min.

Sephacryl S-100 G6 Gel Filtration Chromatography—Fraction IV (361 mg) was dissolved in 5 ml of buffer S and dialyzed against 2 liters of buffer S to an ionic equivalent of 180 mM NaCl. This material was loaded onto a Sephacryl S-100 column (570 ml; 3 cm × 82 cm) equilibrated with buffer S. The χψ activity was eluted with buffer S at 1/15-column volume/h. Active fractions containing at least 50% of the activity of the peak tube were combined to yield fraction V (40 ml, 248 mg). Homogeneous χψ was divided into aliquots, immediately frozen in liquid nitrogen, and stored at −80 °C. This protein is stable frozen for at least 1 year and for 10 days on ice. Table I summarizes the overall purification. Purity of the major fractions was determined by SDS-polyacrylamide gel electrophoresis (Fig. 1).

Physical Characterization of χψ

Determination of the Molar Extinction Coefficient for χψ—The absorption spectrum was determined after a 40-fold dilution of χψ (fraction V) into 10 mM potassium phosphate (pH 7.5) with or without 6 M guanidine HCl. The instrument was blanked by adding a 1/40 volume of buffer S (fraction V buffer) to the same buffers used to dilute χψ. Absorbance, measured from 220–360 nm, reached a maximum at 280 nm. The molar extinction coefficient was calculated using the equations of Edelhoch (1967) (ε280 = Ntrp(5690) + Ntyr(1280)) and the amino acid composition available through the nucleic acid sequence of χ (Carter et al. 1993b) and ψ (Carter et al. 1993a) to calculate the denatured molar extinction coefficient (53,200 liters mol−1 cm−1) and the ratio of the absorbance of native χψ to χψ in 6 M guanidine (0.811) to calculate a molar extinction coefficient of 43,145 liters mol−1 cm−1 for the native protein.

Sedimentation Equilibrium Analysis—To determine the composition, native molecular weight, and equilibrium interactions within the χψ complex, we performed sedimentation equilibrium experiments at 1, 2, and 4 mM χψ. Four equilibrium boundary scans after 84, 88, 92, and 96 h at 35,000 rpm indicated that equilibrium had been achieved (Fig. 2A). From these data, the native molecular weight of the complex was determined using the Beckman IDEAL1 program which can be used to calculate an apparent weight average of single ideal species. If the system contains two or more species sedimenting independently at significant concentrations, then curve fits generated by the IDEAL1 model do not fit the data.

We modeled χψ as a single species sedimenting independently, and as a dimer, trimer, and a tetramer. Only the dimer (1:1 χψ complex) fit the data (Fig. 2B), yielding very low residuals (≤0.02 A280 units) distributed around the theoretical curve. Sedimentation equilibrium data from all three concentrations and each angular velocity were used in close agreement. They provided a native molecular mass for χψ complex of 31,755 ± 178 daltons. The fit for the other models was unacceptable, giving nonrandom residuals that deviated as much as 0.2 A280 units from the theoretical curves (Fig. 2, C–E). Based on the amino acid composition predicted from the DNA sequence and the protein sequence of ψ lacking its amino-terminal methionine, χ and ψ are 16,599 and 15,043 Da, respectively (Xiao et al., 1993a, 1993b). Thus, the species that best represents χψ is a heterodimer of 31,642 Da.

Sedimentation Velocity Analysis—χψ was subjected to sedimentation velocity analysis to further investigate its hydrodynamic properties. The sedimentation and diffusion coefficients were determined using the second moment/boundary spreading method (Muramatsu and Minton, 1988) at 2, 5, and 8 mM χψ. The calculated s20,w value for χψ was 2.6 ± 0.07, independent of protein concentration during sedimentation. The calculated diffusion coefficient (D20,w) was 7.6 × 10−7. The correlation coefficients for both determinations were >0.997. Applying the Svedberg equation, the calculated molecular mass of χψ was 31,400 ± 600 Da. The Stokes radius, 28 ± 0.1 Å, and frictional coefficient, 1.3 ± 0.01 were calculated from D20,w using Stokes’ law and Fick’s law, respectively.

![Fig. 1. Purification of χ and ψ as a complex.](https://example.com/figure1.jpg)

**Table I**

| Fraction | Protein | Activity | Specific activity | Yield |
|----------|---------|----------|------------------|-------|
|          | mg/ml   | mg       | units            | units/mg | %   |
| I. Lysate| 20      | 6500     | 2.5 × 1010       | 3.85 × 108 | 100 |
| II. Ammonium sulfate | 11.6 | 2310 | 2.4 × 1010 | 1.04 × 108 | 100 |
| III. Q-Sepharose | 2.7 | 535 | 1.9 × 1010 | 3.5 × 107 | 76 |
| IV. SP-Sepharose | 2.4 | 361 | 1.3 × 1010 | 3.60 × 107 | 54 |
| V. Sephacryl S-100 | 3.9 | 248 | 9.8 × 106 | 3.95 × 107 | 39.2 |

* The actual yield of χψ from Fraction V was 176 mg as determined using the extinction coefficient.
* The true specific activity of pure χψ is 5.42 × 107 units/mg.
Stimulation of the Reconstituted Holoenzyme Reaction in the Presence of 400 mM Potassium Glutamate—To develop a functional assay for use in monitoring the purification of $\chi^\psi$, we first exploited an earlier observation (Xiao et al., 1993a) that holoenzyme-like activity reconstituted with $\alpha$, $\gamma$, $\delta$, and $\delta'$ becomes more salt-resistant in the presence of $\chi^\psi$. We observed a modest but reproducible stimulation in the presence of $\chi^\psi$ to a reaction using only the $\gamma$ DnaX gene product (Fig. 3A). However, this reaction is much more sensitive than native holoenzyme to glutamate (Griep and McHenry, 1989). Thus, we investigated the effect of the missing component $\tau$.

Holoenzyme reconstituted with the $\tau$ product of the dnaX gene instead of $\gamma$, in the presence of $\chi^\psi$, is extremely resistant to increasing potassium glutamate concentrations up to 800 mM (Fig. 3B). The salt resistance is similar to that observed for native purified holoenzyme$^2$ (Griep and McHenry, 1989). However, in the absence of $\chi^\psi$, the DNA polymerase activity of $\tau$-reconstituted holoenzyme decreased dramatically as a function of increasing potassium glutamate concentration. At 400 mM potassium glutamate, in the presence or absence of $\chi^\psi$, the

$^2$When both $\tau$ and $\gamma$ are present in reconstitution mixes, the activity of the resulting holoenzyme is the same as the $\tau$-reconstituted and native holoenzyme. However, $\gamma$ does not readily enter the holoenzyme assembly under these conditions.
The amount of dNTP incorporation was 190 and 21 pmol, respectively, a 9-fold difference. Thus the \( xzcy \) complex is the key component required for the salt resistance observed in native holoenzyme. This 9-fold dependence for \( xzcy \) in the presence of 400 mM glutamate provided a convenient functional assay to monitor the purification of \( xzcy \) reported in Table I. The assay is linear over a broad range, from 10–50 fmol \( xzcy \) per 25-\( \mu \)l assay (Fig. 4).

**Fig. 3.** \( xzcy \) confers salt resistance to \( \tau \)-reconstituted DNA polymerase III holoenzyme but not \( \gamma \)-reconstituted holoenzyme. DNA synthesis was measured as described under “Experimental Procedures” using holoenzyme reconstituted with \( \tau \) or \( \gamma \) in the presence of the indicated potassium glutamate concentrations. A, each assay contained 600 fmol of pol III core (\( xzcy \) complex), 500 fmol of \( \beta \), 500 fmol of \( \gamma \), 600 fmol of \( \delta \), 600 fmol of \( \delta' \), and 500 fmol of \( xzcy \). B, same as in A, only holoenzyme was reconstituted with 500 fmol of \( \tau \) instead of \( \gamma \). Data represent DNA synthesis by reconstituted holoenzyme in the presence (●) or absence (○) of \( xzcy \). Each data point represents the average of a duplicate determination.

**Fig. 4.** Dependence of DNA polymerase activity of \( \tau \)-reconstituted holoenzyme on \( xzcy \) in 400 mM potassium glutamate. DNA synthesis was measured with DNA polymerase III holoenzyme reconstituted as described under “Experimental Procedures” and in the Fig. 3 legend using \( \tau \) and the indicated amount of \( xzcy \). Each data point represents the average of a duplicate determination.

**Fig. 5.** The presence of \( xzcy \) reduces the level of \( \delta \) and \( \delta' \) required to reconstitute holoenzyme. DNA synthesis was measured as described under “Experimental Procedures” except that 75 fmol of the dnaX gene products (\( \tau \) or \( \gamma \)) were included, and the potassium glutamate concentration was 100 mM. Holoenzyme was reconstituted with \( \gamma \) (●) and \( \tau \) (○) Closed and open symbols represent holoenzyme reconstituted with or without \( xzcy \), respectively. Each data point represents the average of a duplicate determination.

Under Standard Assay Conditions, \( xzcy \) Decreases the Requirement for \( \delta \delta' \) to Physiologically Relevant Levels—We next addressed the effect of \( xzcy \) on the holoenzyme reaction under standard conditions (100 mM glutamate). Under conditions where components have been titrated to saturating levels, \( xzcy \) reproducibly stimulates the reaction ~30%, indicating a small, but real contribution of \( xzcy \) to the intrinsic activity of the complex. However, we observed a requirement for high levels of

\[ 4 \text{Except } \tau \text{ or } \gamma \text{, which was fixed. High levels of } \tau \text{ or } \gamma \text{ (~400 mM)} \]
δδ9 in the absence of χψ (Fig. 5). Nearly 200 nM δδ9 is required to saturate the assay in the absence of χψ. The [δδ9]0 (apparent Kd) or δδ9 dissociation from holoenzyme under these conditions, estimated from the amount required for half-maximal synthesis, is ~40 nM in the absence of χψ. The actual value may be higher, since the high concentrations of some of the reaction components may shift the equilibrium. In any case, these estimates are useful for comparative purposes. In the presence of χψ, the [δδ9]0 and saturation level dropped to 2 and 20 nM, respectively. The cellular levels of most holoenzyme components including the ω polymerase catalytic subunit and the DNA proteins to which χψ binds are about 28 nm6 (Wu et al., 1984; Hawker, 1985). Thus, χψ is presumably required in vivo to permit activation of a significant portion of τ or γ by binding δδ9.

The Major Function of χψ Is to Increase the Affinity of τ and γ for δδ9: BIAcore™ Analysis of χψ Interaction with τ and γ

To further understand the interactions of χψ with τ and γ and to study the effect of χψ on δδ9 binding, we examined the interactions directly in the Pharmacia BIAcore™. This instrument uses the optical phenomenon of surface plasmon resonance to monitor the interaction of an immobilized ligand to a protein in the flow solution that is passed over it (Malmqvist, 1993; Fagerstam et al., 1992) (see the companion study by Dallmann and Mcherry (1995) for more details). Either τ or γ was immobilized to the sensor chip surface, dilute solutions of χψ (15–100 nm) were injected over each, and the binding signal was monitored (plotted as response units which are directly proportional to the mass bound to the chip) (Fig. 6). The buffer used in this analysis contained 100 mM potassium glutamate and the other ionic components of our standard holoenzyme assay. χψ rapidly bound to the τ (Fig. 6) and γ-derivatized sensor chips (data not shown) with nearly equivalent rates (Table I). Passing buffer over the chip permitted us to monitor the first-order dissociation of χψ from the immobilized DNAx protein (Fig. 6). χψ dissociated slowly from both τ and γ (t1/2 = 14–17 min). From the pseudo-first-order rate constant for association and the first-order rate constant of dissociation, we calculated nearly equivalent dissociation constants (1.8–2.5 nm) for both the τ- and γ-χψ interaction. The calculated stoichiometry of τχψ and γχψ was 4:1, using Equation 2 from Dallmann and Mcherry (1995). This is the same stoichiometry reported within the native τ and γ complexes in solution (Dallmann and Mcherry, 1995), indicating that immobilized τ and γ are properly folded and active.

In preliminary BIAcore™ experiments, no high affinity interactions were observed between the following pairs of proteins: τδ, τδ9, γδ, γδ9, or δδ9. With τ or γ coupled to the BIAcore chip, injection of either δ or δ9 (up to 200 nm each) over the coupled DNAx subunit resulted in a signal essentially equal to a control injection over a blank chip. The same result was observed when either δ or δ9 was coupled to the BIAcore™ chip and τ or γ (up to 400 nm each) was injected. Injection of δ over immobilized δ9, or δ9 over immobilized δ also failed to show significant interaction at analyte concentrations up to 2 μM (data not shown). These observations did not rule out possible interactions between these subunit pairs at significantly higher concentrations, but might indicate that the establishment of these pairwise interactions is either kinetically slow or the resulting equilibrium is sufficiently unstable that these interactions do not represent central steps in the holoenzyme assembly pathway. Likewise, χψ (up to 400 nm) also did not appear to interact with either δ or δ9 when they were immobilized on BIAcore™ sensor chips (data not shown).

When equimolar mixtures (200 nm) of χψ + δ or τ + χψ were passed over a δ9-bound chip, no binding was observed (Fig. 7). The small signal observed was due to a refractive index change caused by residual glycerol from the protein storage buffer and is identical to the signal observed from an injection over a blank sensor chip. When a mixture of τ and δ was injected over the δ9 chip, complex formation occurred (Fig. 7). Since neither τ or δ alone binds, the binding observed must represent a highly cooperative assembly of a δδ9 complex. When χψ was injected along with τ and δ, the rate and the extent of binding was greater, indicating that χψ stimulated the rate of binding of δδ9 to τ. Identical binding curves were obtained when γ was used in place of τ or when δ was coupled to the BIAcore™ chip and δ9 was used in the mixture of analyte proteins (data not shown). Due to the complexity of this associating system, no kinetic and equilibrium constants could be obtained since the data could not be fit to the relatively simple binding models

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**TABLE I**

| Immobilized protein | Analyte protein | Association rate constant (10^9 M^-1 s^-1) | Dissociation rate constant (Kd) (nm) | Dissociation constant (Kd) (nm) |
|---------------------|-----------------|------------------------------------------|------------------------------------|----------------------------------|
| χψ                  | χψ              | 3.5 × 10^6                             | 8.7 × 10^-4                        | 2.5 ± 0.4                        |
| τ                   | χψ              | 3.6 × 10^6                             | 6.7 × 10^-4                        | 1.8 ± 0.2                        |

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5 Individual concentrations of δ and δ9 are given.
6 The concentration was calculated based on a cell volume of ~1 fl/cell during balanced growth (Ingraham et al., 1983). One molecule/cell would have a concentration of 1.4 nm.
available in the BIAcore™ evaluation software. Nevertheless, the qualitative conclusion that $\chi$-$\psi$ functions to stabilize the interaction between $\delta$-$\delta'$ and DnaX is consistent with the interpretation of the functional experiments.

**DISCUSSION**

$\chi$ and $\psi$ were purified to homogeneity as a tightly associated complex following overexpression of both subunits from a vector containing an artificial holc-holO operon. We pursued this strategy because of the insolubility of $\psi$ when overproduced individually. Having this material by itself permitted the important demonstration that $\psi$ binds to DnaX and bridges an interaction with $\chi$ (Xiao et al., 1993b), but more detailed biochemical experiments required defined folded material so that interactions could be studied without the complicating step of protein folding in assembly reactions.

$\chi$-$\psi$ when overexpressed as a complex constitutes 15% of the total cellular protein and 11% of the total soluble protein. Together, ammonium sulfate fractionation and Q-Sepharose chromatography yielded nearly pure material. A trace 115-kDa contaminant and smaller molecular mass polypeptides were removed upon SP-Sepharose chromatography. Sephacryl S100 gel filtration chromatography provided only a marginal purification, but ensured that the final material was free of aggregates or unassembled subunits and permitted exchange into a defined buffer. Purified fraction V $\chi$-$\psi$ complex, subjected to polyacrylamide gel electrophoresis, appeared as only two bands of $\sim$15,100 and 16,600 Da even when $40 \mu$g of protein was loaded (Fig. 1, lane 5). Laser densitometry of a Coomassie-stained gel containing $1-10 \mu$g of $\chi$-$\psi$ demonstrated 99% purity.

The molar extinction coefficient of $\chi$ and $\psi$ purified independently were calculated based on the amino acid composition (Xiao et al., 1993a). The sum is the calculated extinction coefficient for the $\chi$-$\psi$ complex 53,200 $\mu$M$^{-1}$. The actual native extinction coefficient is 43,145 $\mu$M$^{-1}$, a 20% difference from the calculated molar extinction coefficient. Use of this rigorously defined extinction coefficient will allow more precision in future experiments.

Sedimentation velocity analysis indicated an $s_{20,W}$ of 2.6, a Stokes radius of 28 Å, a native molecular mass of 31,400 daltons, and a frictional coefficient of 1.3, data that are in reasonable agreement with glycerol sedimentation of $\chi$-$\psi$ (Xiao et al., 1993b) and the sedimentation equilibrium data presented here. The frictional coefficient of 1.3 suggests that $\chi$-$\psi$ is an asymmetric molecule.

Sedimentation equilibrium experiments were conducted to examine the composition of $\chi$-$\psi$ in solution. This technique is particularly powerful because at each position within the boundary established, all components are at sedimentation and chemical equilibrium. The shape of the curve at varying protein concentrations permits a particularly sensitive way to detect multiple molecular species in a mixture and to determine the equilibrium between them. $\chi$-$\psi$ sediments as a single ideal complex and holoenzyme as an assay (Xiao et al., 1993a), but we found that the resulting $\gamma$-reconstituted holoenzyme did not exhibit the salt resistance observed for native holoenzyme (Griep and McHenry, 1989). In a search for conditions that permitted reconstitution of native holoenzyme at high salt levels (400 mM glutamate), we found that both $\tau$ and $\chi$-$\psi$ are required. This result not only provided a convenient linear assay for $\chi$-$\psi$, but also suggested that a $\tau$-$\chi$-$\psi$ interaction occurs within native holoenzyme, providing additional evidence for our model that $\tau$ plays a central role as a clamp loader in holoenzyme (Dallmann et al., 1995; Dallmann and McHenry, 1995).

We examined the influence of $\chi$-$\psi$ on the binding of $\delta$-$\delta'$ to DnaX in the BIAcore™. This instrument permits real-time direct monitoring of the binding of protein in the flow phase to a protein immobilized on a chip. By monitoring binding of $\chi$-$\psi$ to $\tau$ and $\gamma$, we determined a $K_d$ of $\sim$2 nM. That the $K_d$ was roughly equivalent for $\tau$ and $\gamma$ suggests that the site for $\chi$-$\psi$ interaction is entirely within the amino-terminal $\gamma$ domain of $\tau$. The estimated $K_d$ is consistent with the requirement for $\sim$1 nM $\chi$-$\psi$ in our functional assays.

Analyses using the BIAcore™ to monitor DnaX complex formation supported our model of $\chi$-$\psi$ function. $\delta$-$\delta'$ bound to DnaX to a greater extent and more rapidly in the presence of $\chi$-$\psi$ than in its absence. Binding to the BIAcore™ is directly proportional to a change in the amount of mass bound to the chip. Because $\chi$-$\psi$ constitutes 8% of the mass of the $\tau$ complex (Dallmann and McHenry, 1995), the 2-fold increase in binding is not due solely to the mass contribution by $\chi$-$\psi$.

The DnaX complex functions to load the $\beta$ sliding clamp onto DNA. A defined position with the initiation complex that results when pol III core assembles also suggests an elongation role and $\alpha$-DnaX contacts (Reens et al., 1995). The function of $\delta$, $\delta'$, $\chi$, and $\psi$ subunits within the complex remains poorly understood. From the results of this study, we propose that $\chi$-$\psi$, though not required for DNA synthesis per se, is an important component for proper holoenzyme function in vivo. High concentrations of $\delta$-$\delta'$ can overcome most of the $\chi$-$\psi$ requirement in vitro, but concentrations of $\sim$200 nM are required to saturate DnaX under our standard assay conditions (100 mM glutamate). To date, only the $\beta$ subunit is known to be present in excess. Other components of the complex, including $\alpha$ and the DnaX subunits with which $\delta$-$\delta'$ interacts, are present at $\sim$20 copies per cell, which corresponds to $\sim$28 nM. This level of $\delta$-$\delta'$ would permit sequestration of DnaX in a complex in the presence of $\chi$-$\psi$. In the absence of $\chi$-$\psi$, only a fraction of the maximal amount of complex would be formed. The ability of $\chi$-$\psi$ to alter the amount of functional clamp loader present in the cell could also enable $\chi$-$\psi$ to serve a regulatory role or as a modulator of the holoenzyme assembly pathway.

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