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 \( \lambda \) and \( \psi \) subunits of the DNA polymerase III holoenzyme. Normally insoluble by itself, \( \psi \) forms a tight soluble complex with \( \lambda \). A purification procedure that yields pure, active \( \lambda \psi \) complex in 100-mg quantities suitable for biophysical studies is reported. Sedimentation equilibrium studies demonstrate that \( \gamma \psi \) is a 1:1 heterodimer. The presence of \( \lambda \psi \) dramatically lowers the level of \( \delta \delta' \) required to reconstitute holoenzyme to levels expected in vivo. That \( \lambda \psi \) accomplishes this by binding to \( \tau \) and increasing their affinity for \( \delta \delta' \). \( \lambda \psi \) was demonstrated by surface plasmon resonance using a Pharmacia BIACore™ instrument. In the absence of \( \delta \delta' \), \( \lambda \psi \) binds to either \( \gamma \) or \( \tau \) DNAx protein with \( K_d = 2 \text{ nm}^{-1} \).

The DNA polymerase III holoenzyme1 is the replicative polymerase responsible for synthesis of the Escherichia coli chromosome. Holoenzyme is composed of a DNA polymerase III core (\( \alpha \epsilon \theta \)) plus auxiliary subunits that confer the special properties expected of a replicative polymerase (for reviews, see McHenry (1991) and Kuriyan 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) the \( \mu \) forms a sliding clamp that apparently encircles DNA (Kong et al., 1992) and tethers the pol III 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 \( \lambda \psi \) (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; Studewell-Vaughan and O’Donnell, 1991). The ATPase activities of \( \tau \) and \( \gamma \) are stimulated by the presence of \( \delta \delta' \) or \( \lambda \psi \) (Onrust and O’Donnell, 1993, Xiao et al., 1993a), suggesting direct binding of one of the subunits. Gel filtration of mixed subunits shows that \( \delta \delta' \) and \( \lambda \psi \) 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., 1993a). No requirement has been observed for \( \lambda \psi \) 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 \( \gamma \) and \( \psi \) form a 1:1 complex (Xiao et al., 1993b). Gel filtration studies indicated that \( \lambda \psi \) forms a complex with \( \tau \) or \( \gamma \) in solution and that \( \psi \) bridges the interaction of \( \lambda \psi \) with \( \gamma \) (Xiao et al., 1993b).

The insolubility of the \( \psi \) subunit and its tendency to aggregate has limited its utility in physical and functional studies (Xiao et al., 1993a). \( \psi \) required 5 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 \( \lambda \psi \) complex. Explaining an artificial operon that overproduces both \( \lambda \) 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 \( \lambda \psi \) 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.

* The abbreviations used are: holoenzyme, E. coli DNA polymerase III holoenzyme; core pol III, E. coli DNA polymerase III core (\( \alpha \epsilon \theta \)); \( \chi \psi \)-complex, \( \gamma \delta \delta' \chi \psi \)-complex, \( \gamma \delta \delta' \chi \psi \)-DnaX complex, a complex containing either product of the dnaX gene, \( \gamma \) or \( \tau \) with associated \( \delta \delta' \) and \( \chi \psi \); Mes, 2-(N-morpholino)ethanesulfonic acid; SSB, E. coli single-stranded DNA binding protein; pol III, polymerase III; DTT, dithiothreitol.

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χψ Increases the Affinity of DnaX for δ′

EXPERIMENTAL PROCEDURES

E. coli Strains and Growth—The E. coli K-12 strain MC1061 (F−, hsd R2, mcrB1, ara D139 Daru-leu7696 (DEL), Dlac -174, gal U, gal K, rpsL, thi) containing the plasmid pMAF 310 (hdcC+, hol D−, amp R), lac (Q) (Carter et al. 1993a) was grown in a 250-ml fermentor (New Brunswick) in F-media + glucose and ampicillin at 37°C. Cells from a growing 20-liter culture were diluted 1:10 in 180 liters of F-media in the 250-liter fermentor. F-media is composed of yeast extract (14 g liter−1), tryptone (18 g liter−1), KH2PO4 (12 g liter−1), K2HPO4 (1.2 g liter−1) (pH 7.2). Glucose and ampicillin are added to 1% and 50 μg/ml, respectively, at the beginning of the fermentation and at the point of induction with isopropyl-β-D-thiogalactoside (1 mM 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 Sharples 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.

The subunit (I Johnson et al. 1986) and of the τ and χ 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 × 106 units/mg) were purified from overproducing strains as described (Griep and McHenry 1989). DNA polymerase III holoenzyme was partially purified from strains yielding χ subunits by methods developed in this laboratory.2 Bovine serum albumin (RNase-free) used in the buffer for activity assays was purchased from Pharmacia and Amersham Corp., respectively. M13Gori single-stranded DNA (Kaguni and Ray, 1979) was prepared as described by Johnson et al. 1986.

DNA Polymerization Assay—One unit of ψχ was defined as the amount needed to incorporate 1 pmol of (total) nucleotide/min during a 5-min incubation at 30°C into acid-pyridinol DNA under conditions where all other components were saturating. Assays contained 700 fmol of pol II core (4×106 units/mg) purified from overproducing strains as described (Griep and McHenry 1989). DNA polymerase III holoenzyme complexes were purified by chromatography on agarose and by methods developed in this laboratory.2

Protein Determinations—Protein was determined by the Pierce Coomassie plus protein assay reagent according to the manufacturer’s instructions. Bovine serum albumin (fat-free) (Sigma) was used as the standard.

Buffers—These were buffer Q (20% glycerol, 50 mM Tris-HCl (pH 7.8), 1 mM EDTA, 5 mM DTT); buffer SP (20% glycerol, 50 mM Tris-HCl (pH 7.0), 1 mM EDTA, 5 mM DTT); buffer S (20% glycerol, 20 mM potassium phosphate (pH 7.5), 0.5 mM EDTA, 20 mM NaCl, 2.5 mM DTT); buffer HBS (10 mM HEPES-KOH (pH 7.4)), 150 mM NaCl, 3.4 mM EDTA, 0.005% P-20 detergent (Pharmacia Biosensor); and buffer HKGM (50 mM HEPES-KOH, pH 7.4, 100 mM potassium glutamate, 10 mM magnesium acetate, 0.005% P-20 detergent). Potassium glutamate (4 mM) was adjusted to pH 7.5 with KOH.

SDS-Polyacrylamide Gel Electrophoresis—Proteins were separated by electrophoresis according to Laemmli (1970) on an SDS-15% polyacrylamide gel run in a Hoefer vertical gel electrophoresis apparatus for 18 h at 60V. Protein was visualized using overnight staining with a 0.1% solution of Coomassie Brilliant Blue R-250 in 20% methanol and 10% acetic acid, and destaining in a solution of 10% methanol and 10% acetic acid.

Immobilization and Analysis of Proteins on BiAcore® Procedure—Protein-protein interaction studies were performed using a Pharmacia Biosensor BiAcore® instrument. CM5 research grade sensor chips (Pharmacia Biosensor) were used for all experiments. Proteins were immobilized in solutions of buffer HBS at a flow rate of 5 μl/min (20°C). The carboxymethyl dextran matrix of the sensor chip was activated using a 30-μl (6 min) injection of a mixture of 0.2 M 1-ethyl-3-(3-dimethylaminopropyl)-2-carboxyethyl carbodiimide and 0.05 M N-hydroxysuccinimide in water to convert the carboxyl groups of the sensor chip matrix to an N-hydroxysuccinimide ester. This ester is susceptible to nucleophilic attack by amino groups of proteins, resulting in an amide linkage of the protein to the sensor chip. The τ, γ, δ, and δ′ subunits were immobilized to activated sensor chips in buffers and at concentrations optimized for each subunit immobilization conditions were as follows: γ, 50 μg/ml, 5-μl injection, 20 mM sodium phosphate (pH 7.4), δ, 50 μg/ml, 7-μl injection, 20 mM Mes (pH 6.0); and δ′, 100 μg/ml, 30-μl injection, 20 mM Mes (pH 6.0). Unreacted N-hydroxysuccinimide ester groups were quenched by a 30-μl injection of 1 mM ethanolamine-HCl (pH 8.0). Under these conditions, typically 7000 response units (RU) of γ, 3000 RU of δ, and 1500 RU of δ′ were immobilized. Protein-protein interaction studies were carried out in buffer HKGM at a flow rate of 5 μl/min at 20°C.

Analytical Ultracentrifugation—Ultracentrifugation sedimentation velocity and sedimentation equilibrium experiments were performed using a Beckman model XLA analytical ultracentrifuge. All sedimentation experiments were performed at 4°C using a Beckman four-hole An-60Ti rotor and were carried out in 1.2-m path length double-sector cells with quartz windows. The protein absorbance was monitored at 230 nm and 280 nm. ψχ complex (fraction V) was dialyzed overnight against 3 liters of 5% glycerol, 100 mM NaCl, 2 mM EDTA, 20 mM Tris (pH 7.5), and 1 mM EDTA to a final concentration of 57 μM. The sample contained ψχ, 1% NaCl (2 mM EDTA, 0.1 mM Tris-HCl (pH 7.5) and 1 mM EDTA), and the blank container contained buffer only. For sedimentation equilibrium studies, six-channel cells were used. Here, the ψχ concentrations were 4 mM, 2 mM, and 1 mM, and radial absorbance scans were taken at 4-h intervals over 95 h (total) at 15,000, 25,000, and 35,000 rpm. The data were subjected to the single data set analysis model, “IDEAL 1.” For sedimentation velocity analysis, the ψχ concentrations were 8, 4, and 2.5 μM and scans were taken at 30-min intervals over 20 h at 40,000 rpm. Data were subjected to analysis using the second moment/boundary spreading method in the XLA-VELOC program.

RESULTS

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

Overproduction and Purification of ψχ

Preparation of Cell Lysate and Ammonium Sulfate Precipitation—All operations used in the purification of ψχ 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 millilitre (45% saturation) was added to the resulting supernatant, and precipitant (fraction II) was collected by centrifugation at 22,000 × g for 60 min. Densitometry of Coomassie-stained SDS-polyacrylamide gels indicated ψ and χ 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 ml) was dissolved in 60 ml of buffer Q and dialyzed against a total of 3 liters of buffer Q to anionic equivalent of 20 mM NaCl (buffer Q = 20 mM NaCl). This material was loaded onto a Q-Sepharose column (750 ml; 5.8 cm × 30 cm) equilibrated with buffer Q at 0.3-column volume/h. The ψχ 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

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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 0.5-column volume/h. The χψ activity was eluted with an 8-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 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 (82 cm) equilibrated with buffer S. The χψ activity was eluted with buffer S 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.

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 equation of Edelhoch (1967) (ε280 = Ntrp(5690) + Ntrp(1280)). We used 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 μM 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 in close agreement. They provided 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; Carter et al., 1993a, 1993b). Thus, the species that best represents χψ is a heterodimer of χ(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 μM 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.

**Table 1**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein</th>
<th>Activity</th>
<th>Specific activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Lysate</td>
<td>20</td>
<td>6500</td>
<td>2.5 × 10¹⁰</td>
<td>3.85 × 10⁹</td>
</tr>
<tr>
<td>II. Ammonium sulfate</td>
<td>116.6</td>
<td>2310</td>
<td>2.4 × 10¹⁰</td>
<td>1.04 × 10⁹</td>
</tr>
<tr>
<td>III. Q-Sepharose</td>
<td>2.7</td>
<td>353</td>
<td>1.9 × 10¹⁰</td>
<td>3.5 × 10⁹</td>
</tr>
<tr>
<td>IV. SP-Sepharose</td>
<td>2.4</td>
<td>361</td>
<td>1.3 × 10¹⁰</td>
<td>3.60 × 10⁹</td>
</tr>
<tr>
<td>V. Sephacryl S-100</td>
<td>3.9</td>
<td>248</td>
<td>9.8 × 10⁹</td>
<td>3.95 × 10⁹</td>
</tr>
</tbody>
</table>

*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 \( \alpha-e, \gamma, \delta \), 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 (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

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Footnote: 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.
amount of dNTP incorporation was 190 and 21 pmol, respectively, a 9-fold difference. Thus the χψ complex is the key component required for the salt resistance observed in native holoenzyme. This 9-fold dependence for χψ in the presence of 400 mM glutamate provided a convenient functional assay to monitor the purification of χψ reported in Table I. The assay is linear over a broad range, from 10–50 fmol χψ per 25-μl assay (Fig. 4).

Under Standard Assay Conditions, χψ Decreases the Requirement for δδ' to Physiologically Relevant Levels—We next addressed the effect of χψ on the holoenzyme reaction under standard conditions (100 mM glutamate). Under conditions where components have been titrated to saturating levels, 4 χψ reproducibly stimulates the reaction ~30%, indicating a small, but real contribution of χψ to the intrinsic activity of the complex. However, we observed a requirement for high levels of

4 Except τ or γ, which was fixed. High levels of τ or γ (~400 nM) decrease the levels of δδ' required, presumably by mass action effects.
\( \delta \delta' \) in the absence of \( \chi' \psi \) (Fig. 5). Nearly 200 nM \( \delta \delta' \) is required to saturate the assay in the absence of \( \chi' \psi \). The \( [\delta \delta']_{20} \) (apparent \( K_d \)) or \( \delta \delta' \) dissociation from holoenzyme under these conditions, estimated from the amount required for half-maximal synthesis, is \( \approx 40 \) nM in the absence of \( \chi' \psi \). 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 \( \chi' \psi \), the \( [\delta \delta']_{20} \) and saturation level dropped to 2 and 20 nM, respectively. The cellular levels of most holoenzyme components including the \( \omega \) polymerase catalytic subunit and the DnaX proteins to which \( \chi' \psi \) binds are about 28 nM (Wu et al., 1984; Hawker, 1985). Thus, \( \chi' \psi \) is presumably required in vivo to permit activation of a significant portion of \( \tau \) or \( \gamma \) by binding \( \delta \delta' \).

The Major Function of \( \chi' \psi \) Is to Increase the Affinity of \( \tau \) and \( \gamma \) for \( \delta \delta' \). BIAcore\textsuperscript{TM} Analysis of \( \chi' \psi \) Interaction with \( \tau \) and \( \gamma \)

To further understand the interactions of \( \chi' \psi \) with \( \tau \) and \( \gamma \) and to study the effect of \( \chi' \psi \) on \( \delta \delta' \) binding, we examined the interactions directly in the Pharmacia BIAcore\textsuperscript{TM}. 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 (Malmbquist, 1993; Fagerstam et al., 1992) (see the companion study by Dallmann and Mchenery (1995) for more details). Either \( \tau \) or \( \gamma \) was immobilized to the sensor chip surface, dilute solutions of \( \chi' \psi \) (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 nM potassium glutamate and the other ionic components of our standard holoenzyme assay. \( \chi' \psi \) rapidly bound to the \( \tau \) (Fig. 6) and \( \gamma \)-derivatized sensor chips (data not shown) with nearly equivalent rates (Table II). Passing buffer over the chip permitted us to monitor the first-order dissociation of \( \chi' \psi \) from the immobilized DnaX protein (Fig. 6). \( \chi' \psi \) dissociated slowly from both \( \tau \) and \( \gamma \) (Fig. \( a \), 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 \( \tau \)- and \( \gamma \)-derivatized proteins. This is the same stoichiometry reported within the native \( \tau \) and \( \gamma \) complexes in solution (Dallmann and Mchenery, 1995), indicating that immobilized \( \tau \) and \( \gamma \) are properly folded and active.

In preliminary BIAcore\textsuperscript{TM} experiments, no high affinity interactions were observed between the following pairs of proteins: \( \tau \delta \), \( \tau \delta' \), \( \gamma \delta \), or \( \gamma \delta' \). With \( \tau \) or \( \gamma \) coupled to the BIAcore chip, injection of either \( \delta \) or \( \delta' \) (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 \( \delta \) or \( \delta' \) was coupled to the BIAcore\textsuperscript{TM} chip and \( \tau \) or \( \gamma \) (up to 400 nM each) was injected. Injection of \( \delta \) over immobilized \( \delta' \), or \( \delta' \) over immobilized \( \delta \) also failed to show significant interaction at analyte concentrations up to 2 \( \mu \)M (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, \( \chi' \psi \) (up to 400 nM) also did not appear to interact with either \( \delta \) or \( \delta' \) when they were immobilized on BIAcore\textsuperscript{TM} sensor chips (data not shown).

When equimolar mixtures (200 nM) of \( \chi' \psi \) + \( \delta \) or \( \tau + \chi' \psi \) were passed over a \( \delta' \)-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 obtained from an injection over a blank sensor chip. When a mixture of \( \tau \) and \( \delta \) was injected over the \( \delta' \)-bound chip, complex formation occurred (Fig. 7). Since neither \( \tau \) or \( \delta \) alone binds, the binding observed must represent a highly cooperative assembly of a \( \delta \delta' \) complex. When \( \chi' \psi \) was injected along with \( \tau \) and \( \delta \), the rate and the extent of binding was greater, indicating that \( \chi' \psi \) stimulated the rate of binding of \( \delta \delta' \) to \( \tau \). Identical binding curves were obtained when \( \gamma \) was used in place of \( \tau \) or when \( \delta \) was coupled to the BIAcore\textsuperscript{TM} chip and \( \delta' \) 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.
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 holoC-holD 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 \(-15,100 \) and \( 16,600 \) Da even when \( 40 \mu \text{g} \) of protein was loaded (Fig. 1, lane 5). Laser densitometry of a Coomassie-stained gel containing \(-10 \mu \text{g} \) 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 \( \text{m}^{-1} \). The actual native extinction coefficient is 43,145 \( \text{m}^{-1} \), 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 A, 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 species with native molecular mass of 31,755 Da, a difference of less than 1% from the calculated molecular mass for a 1:1 heterodimer. Although it is possible that \( \chi \psi \) sediments independently as monomers, or as a trimer or tetramer, these curve fits do not correlate with the data generated by sedimentation equilibrium studies (Fig. 2).

Holoenzyme activity can be reconstituted without \( \chi \psi \) under optimal levels of the other holoenzyme subunits. Thus, to develop a functional assay, we needed to define a set of conditions that provided a maximal stimulation by \( \chi \psi \). We reproduced the findings of O’Donnell and colleagues that a modest \( \chi \psi \) requirement exists at elevated salt concentrations using reconstitution of \( \gamma \) 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 \( \tau \psi \) 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 \( \psi \) 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 \( \chi \) to \( \tau \) and \( \psi \), we determined a \( K_d \) of \(-2 \text{ nM} \). That the \( K_d \) was roughly equivalent for \( \tau \) and \( \psi \) suggests that the site for \( \psi \) interaction is entirely within the amino-terminal \( \gamma \) domain of \( \tau \). The estimated \( K_d \) is consistent with the requirement for \(-1 \text{ nM} \) \( \chi \psi \) 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 on the BIACore™ is directly proportional to a change in the amount of mass bound to the chip. Because \( \psi \) 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 \( \psi \).

The DNA 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 requirement and \( \alpha \)-DnaX contacts (Reems 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 \( \psi \), 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 \(-200 \text{ nM} \) 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 \(-20 \) copies per cell, which corresponds to \(-28 \text{ nM} \). 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.

REFERENCES

Hawker, J. (1985) Master’s thesis, University of Texas Graduate School of Medical Sciences, Houston, TX

$\chi^\psi$ Increases the Affinity of DnaX for $\delta\delta'$