The unstructured C-terminus of the τ subunit of *Escherichia coli* DNA polymerase III holoenzyme is the site of interaction with the α subunit

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ABSTRACT

The τ subunit of *Escherichia coli* DNA polymerase III holoenzyme interacts with the α subunit through its C-terminal Domain V, τ_C16. We show that the extreme C-terminal region of τ_C16 constitutes the site of interaction with α. The τ_C16 domain, but not a derivative of it with a C-terminal deletion of seven residues ($\tau_C16\triangle7$), forms an isolable complex with α. Surface plasmon resonance measurements were used to determine the dissociation constant (K_D) of the $\alpha-\tau_C16$ complex to be ~260 pM. Competition with immobilized τ_C16 by τ_C16 derivatives for binding to α gave values of K_D of 7 μM for the $\alpha-\tau_C16\triangle7$ complex. Low-level expression of the genes encoding τ_C16 and $\tau_C16\triangle7$, but not $\tau_C16\triangle11$, is lethal to *E. coli*. Suppression of this lethal phenotype enabled selection of mutations in the 3′ end of the τ_C16 gene, that led to defects in α binding. The data suggest that the unstructured C-terminus of τ becomes folded into a helix–loop–helix in its complex with α. An N-terminally extended construct, τ_C24, was found to bind DNA in a salt-sensitive manner while no binding was observed for τ_C16, suggesting that the processivity switch of the replisome functionally involves Domain IV of τ.

INTRODUCTION

DNA polymerase III holoenzyme (Pol III HE), the enzyme responsible for *Escherichia coli* chromosomal DNA synthesis, is a complex of 10 different polypeptides (1–7). It is comprised of three functionally distinct but interconnected subassemblies: the core polymerase, the sliding clamp and the clamp loader complex (8).

There are two core polymerases in the HE, each consisting of three different subunits (9): α, the DNA polymerase (10); ε, the proofreading exonuclease (11); and θ, which has a role in stabilizing ε (12). The core polymerase is neither highly processive nor rapid in DNA synthesis: it polymerizes nucleotides at a rate of ~20/s (10) and dissociates from DNA after incorporating 10–20 nt (13). The β sliding clamp subunit, needed for rapid and highly processive DNA synthesis (14), is a ring-shaped head-to-tail dimer (15). Once it is assembled onto DNA by the clamp loader complex, interaction of β_2 with the α subunit confers efficient synthesis on all core polymerase subassemblies (16).

The single clamp loader within Pol III HE contains seven subunits, with composition $\tau_2\gamma\delta\delta'\chi\psi$ (17). It hydrolyzes ATP in a DNA-dependent manner to load β_2 clamps onto DNA for interaction with both core polymerases (18–21). The χ and ψ subunits are involved in binding to ssDNA-binding protein (SSB) (22) and participate in the primase-to-polymerase switch on the lagging strand (23). In an interaction modulated by δ′, the δ subunit binds to β_2 (24), inducing a conformational change in the clamp and subsequent opening of the β_2 ring (25).

The three ATP motor subunits of the clamp loader (γ and the two τ subunits) are encoded by the same gene, *dnaX* (26,27). The 71-kDa τ subunit (28) is the full-length *dnaX* product whereas γ (47 kDa) is a truncated form produced as the result of a programmed translational frameshift (29–31). The γ subunit and the N-terminal portions of the two τ subunits bind δ and δ′, forming a circular pentamer that functions as the clamp loader (32,33).

The holoenzyme contains two αεθ core polymerases to enable simultaneous replication of both the leading and the lagging strands (34). These and the clamp loader are held together by the two τ subunits (35) via the strong $\alpha-\tau$ interaction (34). Deletion of 48 residues from the

C-terminus of α (residues 1113–1160) eliminates its binding to τ, while removal of 705 residues or more from the N-terminus also has a large effect on τ binding. (36). While this may indicate there are two regions of α that contact τ, the involvement of the N-terminal domains of α might be indirect through stabilization of the C-terminal region or through conformational changes that occur during function of the complex. Indeed, there appear to be two different binding modes for the α–τ interaction (37–39) depending on whether or not the holoenzyme is bound to a primer-template DNA (39).

As shown in Figure 1A, the τ subunit has a five-domain structure (40), the N-terminal Domains I–III being identical to γ. The unique 24-kDa C-terminal fragment comprising most of Domain IV and all of Domain V (residues
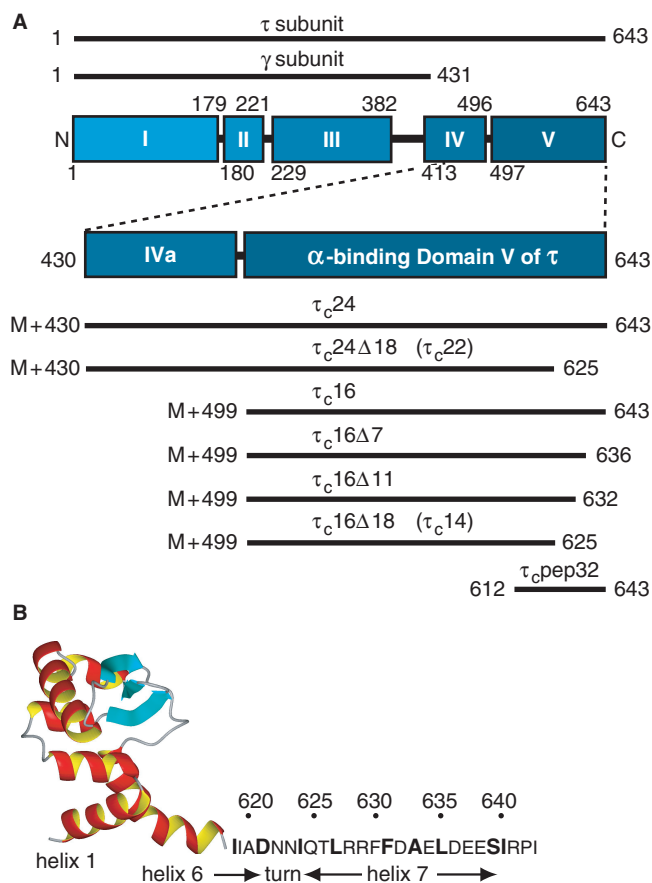

Figure 1. Domain structure of the τ subunit of *E. coli* DNA polymerase III holoenzyme. (**A**) τ is comprised of five domains; domain boundaries are indicated by residue numbers. Domains I–III are shared with the γ subunit, while most of the DnaB-binding Domain IV and all of the α-interacting Domain V (τ_C16) are unique to τ. Schematic representations of τ_C24 and τ_C16 derivatives used in this work are shown below; M+ indicates that τ_C24 and τ_C16 and derivatives constructed by deletion of the indicated numbers of residues from the unstructured C-terminus contain an N-terminal methionine introduced during cloning, followed by the indicated residues of τ. (**B**) Solution structure of the structured core of Domain V (residues Pro507–Ser617 of τ) determined by NMR (43). The sequence of the unstructured region at the C-terminus (43,44) is given in single-letter code, with highly conserved residues (43) marked with larger font. Secondary structural elements that may be induced in this region on interaction with α are indicated below. Part of the figure was drawn using MOLMOL (64).

430–643; referred to in this article as τ_C24) is connected to Domain III by a proline-rich tether that may be flexible (38). The τ_C24 protein can be isolated in monomeric form (41), and is reported to bind both to primed DNA (38) and to a 20-mer peptide from the C-terminus of α in an interaction modulated by DNA structure (39). The 8-kDa N-terminal region of τ_C24 (termed Domain IVa, residues 430–498 of τ) is responsible for binding to DnaB helicase (42), and the 16-kDa C-terminal domain (Domain V; residues 499–643, here also referred to as τ_C16) binds to α (40).

The solution structure of the folded core of Domain V of *E. coli* τ, a 14.3-kDa protein (called τ_C14) derived from τ_C16 by deletion of 18 C-terminal residues, has been determined by NMR spectroscopy (Figure 1B), and is reported in the accompanying article (43). Given that recent NMR studies (43,44) have also shown that all N- and/or C-terminal residues additional to the τ_C14 core in the longer protein constructs τ_C24, τ_C22 (τ_C24 lacking the C-terminal 18 residues), and τ_C16 (see Figure 1A) are intrinsically unstructured, we can now develop a near-complete picture of the structure of the τ subunit in which the structured parts of Domains I–III and V are linked together by flexible segments that provide both docking sites for interactions with other proteins and DNA, as well as flexibility to enable structural transitions in the replisome as it cycles through its various functional states.

In this article, we describe the genesis of the τ_C14 fragment and show that the additional residues in τ_C16 relative to τ_C14 are required for formation of an isolable complex with α. Alignment and secondary structure prediction combined with point mutagenesis and deletion studies suggest the induction of α-helices within the otherwise flexible (44) C-terminal region of τ_C16 upon its interaction with α. We also show that although both τ_C22 and τ_C24 proteins bind weakly through predominantly electrostatic interactions to single-stranded (ss), double-stranded (ds) and primed DNA, τ_C16 does not. This localizes the putative DNA-interaction site in τ_C24 to Domain IVa. Implications for the role of this region of τ in switching processivity in Pol III HE are discussed.

MATERIALS AND METHODS

Plasmids

Full details of construction of plasmids are given in the Supplementary Data. Plasmids pPT153 and pJC490 that direct overproduction of the *E. coli* *dnaX* gene product(s) under control of tandem phage λ p_R and p_L promoters were as described (45). Plasmid pZT3(1G4G), which directs synthesis of the τ subunit of Pol III HE in the complete absence of γ, was a gift of Dr Zenta Tsuchihashi (46). A 1151-bp NdeI–NheI fragment of the *dnaX* gene was isolated from pZT3(1G4G) and ligated between the corresponding sites in pJC490 to give pJC491 (Supplementary Figure S1). As expected, this plasmid directed high-level overproduction of τ, but not γ, and has been used by us for overproduction of full-length τ (data not shown).

To construct pSH1062, designed to direct production of τ_C16 under control of a phage T7-promoter, a PCR was used with pPT153 as a template and primers designed to amplify the region of *dnaX* between the codon for Lys499 and the TGA stop codon. An ATG start codon was inserted immediately before codon 499 as part of an NdeI site and the stop codon was followed immediately by an EcoRI site. The NdeI–EcoRI fragment was inserted between the corresponding sites of pETMCSI (47). The same fragment was subsequently isolated from pSH1062 and inserted between the same sites in the λ-promoter vector pND706 (48) to give pSJ1064 (Figure S2). A similar strategy was used to construct plasmid pSJ1308, which directs production of τ_C14 in pND706, except that a TAA stop codon was inserted after the codon for Gln625 of τ, and pJC491 was used as a template for PCR amplification of the NdeI–EcoRI fragment (Figure S3). To construct T7-promoter plasmids pSJ1318 and pSJ1319 that direct production of $\tau_C16\Delta11$ and $\tau_C16\Delta7$, respectively, appropriate complementary oligonucleotide pairs were used to replace the 93-bp BssHII–EcoRI fragment at the 3′ end of the gene encoding τ_C16 in pSH1062. The NdeI–EcoRI fragment encoding $\tau_C16\Delta7$ was subsequently isolated from pSJ1319 and inserted between the same sites in pND706 (48) to yield pSJ1326.

Similar strategies were used to construct pSJ1330, a λ-promoter plasmid that directs overproduction of τ_C24 (N-terminal Met followed by Lys430–Ile643 of τ) and pKO1297, a T7-promoter plasmid for overproduction of τ_C22 (N-terminal Met plus Lys430–Gln625 of τ). Because the mRNA stem-loop structure normally involved in translational frameshifting in *dnaX* expression affected expression of the truncated genes, silent mutations were introduced to disrupt mRNA secondary structure without changing the amino acid sequence of the proteins (see Supplementary Data for details).

For biotinylation of τ_C14 and τ_C16, we first constructed a T7-promoter vector pKO1274 by insertion of two complementary oligonucleotides that encode the biotin-tag sequence MAGLNDIFEAQKIEWHEH (49) between the NdeI and MluI sites of pETMCSI (47). This operation resulted in elimination of the first NdeI site upstream of the biotin-tag sequence and allows fusion of a gene in-frame between unique NdeI and EcoRI sites to generate genes that encode N-terminally biotin-tagged proteins. NdeI–EcoRI fragments encoding τ_C14 and τ_C16 from pSJ1308 and pSJ1064 were then inserted into pKO1274 to yield pKO1282 and pKO1283, respectively. The complete bio-τ_C16 gene in pKO1283 was then isolated on a 527-bp XbaI–EcoRI fragment and inserted between corresponding sites in the λ-promoter vector pND706 (48) to yield pKO1294. For overexpression of N-terminally His$_6$-tagged *E. coli* biotin ligase, the *birA* gene was amplified by PCR using *E. coli* AN1459 (50) chromosomal DNA as a template, and inserted as a 972-bp NdeI–EcoRI fragment between corresponding sites in the T7-promoter vector pETMCSIII (47) to yield pKO1298.

Nucleotide sequences of all oligonucleotide-derived and PCR-generated fragments inserted into vectors were confirmed by the Biomolecular Resource Facility, Australian National University.

E. coli strains

Strain AN1459 (50) was used routinely as host during plasmid construction. Strains used for protein expression included the *lacP-T7 gene 1$^+$* strains BL21(λDE3)*recA* (51) and BL21(λDE3)/pLysS (52).

Targeted mutagenesis by error-prone PCR

Plasmid pSH1062 was used as a template for error-prone PCR amplification of the gene encoding τ_C16. The PCR product was generated using a primer corresponding to a DNA sequence preceding the unique BssHII site within the gene (5′-CGGTTGAACTGACTATC GTTGAAGATG) and another complementary to a sequence following the stop codon and unique EcoRI site in the vector (5′-CCTTTCGGGCTTTGTTAGCAG). To generate mutations, Taq DNA polymerase was used in the presence of a blend of 3.3 (6 libraries) or 6.6 mM MnCl$_2$/MgCl$_2$ (6 libraries). Each set of libraries, consisting of two separate reaction mixtures that were subsequently pooled, respectively had 3.0, 4.5, 6.0, 7.5, 9.0 and 15.0 mol% of MnCl$_2$ in the Mn^{2+}–Mg^{2+} mixture. PCR products were isolated from an agarose gel following digestion with BssHII and EcoRI. Libraries of mutagenized fragments (93 bp) were ligated between the same restriction sites in pSH1062 and transformed into strain BL21(λDE3)*recA*. Transformants were selected for growth on LB plates containing 100 μg/ml ampicillin at 37°C. Plasmids were isolated from 32 randomly picked colonies, including representatives from all of the mutant libraries, and the nucleotide sequences of the mutant genes were determined. Eight plasmids did not yield interpretable sequences; the gene encoding τ_C16 in the remaining 24 plasmids all contained mutations that altered the amino acid sequence of the protein in the targeted region (see Results section). The six plasmids (pSJ1320–pSJ1325) that contained single point mutations, which respectively direct overproduction of mutant τ_C16 proteins S617P, I618T, L627P, F631I, L635P and D636G, were used for isolation of proteins for further study.

Proteins and peptides

The α subunit of Pol III was purified as described previously (53). A representative method for purification of τ_C domains (τ_C16, method B) is given below (see also Figure S4), while details of preparation of highly purified samples of τ_C24, τ_C22, τ_C16 (method A), τ_C14 (Figure S5), bio-τ_C16, bio-τ_C14, τ_C16 truncation and point mutants and biotin ligase (54) are described in the Supplementary Data. Protein concentrations were determined spectrophotometrically at 280 nm, using calculated values of ε_{280} (55): α subunit, 95 440 M^{-1} cm^{-1}; τ_C24 and τ_C22, 25 420 M^{-1} cm^{-1}; τ_C14, $\tau_C16\Delta11$, $\tau_C16\Delta7$, τ_C16 and point mutants, 18 350 M^{-1} cm^{-1}; bio-τ_C14, bio-τ_C16, 24 040 M^{-1} cm^{-1}. Molecular weights of all purified proteins were confirmed by ESI-MS using a VG Quattro II mass spectrometer with samples that had been dialyzed extensively into 0.1% formic acid containing 1 mM 2-mercaptoethanol (see Supplementary Data). Peptides were synthesized using standard solid-phase methods at

the Biomolecular Resource Facility, Australian National University, purified by reverse-phase HPLC and lyophilized. Their composition and purity was confirmed by MALDI-MS, and solutions were prepared by weight.

Purification of τ_C16 (method B)

Escherichia coli strain BL21(λDE3)*recA*/pSJ1064 was grown at 30°C in LB medium containing ampicillin (100 mg/l) to $A_{595} = 1.0$, then overproduction of τ_C16 was induced at 42°C (2.5 h). Cells were harvested by centrifugation (11 000 × *g*; 5 min), frozen in liquid N_2 and stored at −70°C. After thawing, cells (8 g from 3 l of culture) were resuspended in buffer A [50 mM Tris.HCl (pH 7.6), 1 mM EDTA, 1 mM dithiothreitol] containing 20 mM spermidine (120 ml). Proteinase inhibitor cocktail 'Complete' pills (Roche Diagnostics; 2 pills) were added together with 0.7 mM phenylmethanesulfonyl fluoride just prior to the cells being lysed with a French press operated at 12 000 p.s.i. The lysate was clarified by centrifugation (35 000 × *g*; 30 min) to yield the soluble Fraction I. Proteins that were precipitated from Fraction I by addition of solid ammonium sulfate (0.36 g/ml) and stirring for 60 min were collected by centrifugation (35 000 × *g*; 30 min) and dissolved in buffer A supplemented with 150 mM NaCl (40 ml). The solution was dialyzed against 3 changes of 2 l of the same buffer, to yield Fraction II. Fraction II was applied at 2 ml/min to a column (2.5 × 16 cm) of Toyopearl DEAE-650M resin that had been equilibrated in the same buffer. Fractions containing proteins that did not bind to the column were pooled and dialyzed against 3 changes of 2 l of buffer A. The dialysate (Fraction III, 50 ml) was loaded at 2 ml/min onto a column (2.5 × 16 cm) of the same resin, now equilibrated in buffer A. After the column had been washed with 200 ml of buffer A, τ_C16 was eluted using a linear gradient (360 ml) of 0–150 mM NaCl in buffer A. It eluted in a single peak at ∼25 mM NaCl. Fractions containing highly purified τ_C16 were pooled and dialyzed against three changes of 2 l of 20 mM Tris.HCl (pH 7.6), 0.5 mM EDTA, 1 mM dithiothreitol, 150 mM NaCl (buffer GF), to give Fraction IV (23 ml, containing 54 mg of protein). The purity of the protein was assessed at each step by SDS-PAGE (see Supplementary Data).

Protein–protein interaction analysis by gel filtration

Gel filtration analysis of interactions between α and τ_C16, τ_C14, $\tau_C16\Delta7$, $\tau_C16\Delta11$ and τ_C16 point mutants were carried out at 4°C using a column (1 × 40 cm) of Sephacryl S100 HR (Amersham Biosciences) equilibrated in buffer GF and flowing at 0.5 ml/min. Excess τ_C protein was mixed with α (see below) in buffer GF for 10 min at 4°C prior to loading. Unless stated otherwise, proteins in collected fractions (500 μl) were precipitated by addition of 2 ml of ice-cold acetone at 0°C. After 15 min, protein pellets were collected by centrifugation (20 000 × *g*; 15 min) and dried for 10 min in air at room temperature prior to being dissolved in 40 μl of loading buffer and separated by electrophoresis in 15% SDS-PAGE gels that were stained with Coomassie blue. For these analyses, 35 μM α was mixed with 85 μM τ_C16 in 120 μl of buffer, or

55 μM α with 250 μM τ_C14 in 75 μl of buffer. Analysis of interaction between α and the other proteins used mixtures of 15–20 μM α and 60–80 μM τ_C protein in 150 μl of buffer.

Protein–protein interaction analysis by SPR

A BIACORE 2000 instrument was used to study interactions of α with bio-τ_C16 in the absence (direct assay) or presence of competitor proteins and peptides (competition assay). All experiments were carried out at 20°C in surface plasmon resonance (SPR) buffer [50 mM Tris.HCl (pH 7.6), 1 mM EDTA, 1 mM dithiothreitol, 0.3 M NaCl, 0.005% surfactant P20].

For the direct assay, a streptavidin-coated sensor chip (SA chip; Biacore) was activated with three sequential injections of 1 M NaCl, 50 mM NaOH (1 min each at 5 μl/min). A solution of 1 nM bio-τ_C16 in SPR buffer was immobilized to yield an increase of 30 response units (RUs) on one flow cell. Another flow cell was unmodified and served as a control. Binding studies were carried out at a flow rate of 30 μl/min in a carefully designed SPR buffer to completely eliminate non-specific binding of α to the chip surface. Solutions of α (0–3 nM) were made to flow during 500 s. After 5000 s of dissociation, the flow cells were regenerated with a 1 min injection of 4 M MgCl$_2$ at 5 μl/min. This completely removed α from the surface of the chip. Sensorgrams were fit globally to a 1:1 Langmuir binding with mass transfer model, using BIAevaluation 3.1 software (Biacore). A similar flow cell with 30 RU of immobilized bio-τ_C14 was used for a similar experiment with concentration of α as high as 1 μM.

Similar flow cells (15–20 RU of bio-τ_C16) were used for the competitive binding assays; α alone (1 nM) was first injected as described above, and dissociation monitored over 4000–5000 s to give values of the association (k_a) and dissociation rate constants, k_d and hence K_D ($=k_d/k_a$). Mixtures of 1 nM α with a competitive inhibitor (*I*) of the interaction at 3 or 4 concentrations in an appropriate pre-determined range, were then injected, and sensorgrams were recorded as described above except that dissociation was monitored over 500 s. Each curve was fit separately (1:1 Langmuir binding model with mass transfer correction) to determine values of $k_{a,app}$. Since k_d was shown not to be affected by the presence of *I*, $k_d/k_{a,app}$ gave values of the apparent dissociation constant $K_{D,app}$ at each [*I*]. In this analysis, we assumed simple competitive inhibition by *I* of α binding to bio-τ_C16, so the value of R_{max} (computed RU at saturation with 1 nM α) was also fixed for all sensorgrams within each experiment. Under these circumstances, the relationship between $K_{D,app}$ and [*I*] is given by:

$$K_{D,app} = K_D(\alpha - \tau_C16) \cdot \left(1 + \frac{[I]}{K_D(\alpha - I)}\right). \qquad \mathbf{1}$$

Thus, a plot of $K_{D,app}$ versus [*I*] should be linear with intercept $c = K_D(\alpha - \tau_C16)$ and slope $m = K_D(\alpha - \tau_C16)/K_D(\alpha - I)$ from which $c/m = K_D(\alpha - I)$.

Protein–DNA interaction analysis by SPR

Interactions of τ_C domains with 5′-biotinylated ss and ds oligonucleotides and a DNA primer-template structure immobilized on an SA chip were studied with the BIACORE instrument in binding buffer, 10 mM Na.HEPES, pH 7.4, containing 0.005% surfactant P20. The 21-mer ssDNA was 5′-bio-$(dP)_{10}$-CTTTAGTTAC AACATACTTAT-3′, where 5′-bio represents the site of biotinylation and $(dP)_{10}$ represents a 10-mer abasic deoxyribosephosphate spacer (56). The 21-bp ds-DNA was comprised of the same oligonucleotide annealed to a perfectly complementary 21 mer, while the primer-template comprised the same 5′-biotinylated 21-mer annealed to a partially complementary 36-mer: 5′-AGCA GCCAGCTCCGAATAAGTATGTTGTAACTAAAG. An SA chip (Biacore) was activated as described above. A solution of 2 nM of the 5′-biotinylated ssDNA in binding buffer containing 150 mM NaCl was first used to immobilize ∼100 RU of ssDNA onto each of the surfaces of flow cells 2−4 (6 min each at a flow rate of 5 µl/min), while flow cell 1 was left unmodified and served as a control. Then, solutions of 1 µM of each of the complementary DNAs were used separately to prepare the surfaces of flow cells 3 and 4 with ds and primer-template DNA by hybridization (2 min at 5 µl/min).

Binding studies were carried out at a flow rate of 20 µl/min in binding buffer with various [NaCl] from 25 to 150 mM. Solutions of 1 µM τ_C24, τ_C22 and τ_C16 prepared by ∼100-fold dilution in buffers with NaCl were coinjected for 180 s following the 60 s injection of the buffer used for protein dilution. Flow cells were regenerated with 1 min injections of 1 M $MgCl_2$ at 5 µl/min.

RESULTS

Overproduction and proteolysis of τ

The plasmid pJC491 that directs overexpression of full-length τ under control of tandem temperature-inducible phage λ p_R and p_L promoters was constructed by insertion of a fragment of the *dnaX* gene bearing silent A to G mutations in codons 428 and 429 (46) into the *dnaX*$^+$ plasmid pJC490 (45). These mutations suppressed translational frameshifting, so that τ could be produced without significant production of γ (not shown). Nevertheless, in early attempts to purify τ from an *ompT*$^+$ strain (AN1459) containing pJC490, proteolysis was observed to produce fragments of 47 kDa (N-terminal fragment similar in size to γ), 24 and 16 kDa, and these products could be enriched by overnight storage of cell lysates at 4°C prior to purification. We used this strategy to generate the three fragments and purified both of the smaller fragments by chromatography (not shown). They were identified from their molecular weights determined by ESI-MS to correspond to the Domain V (16.4 kDa) and Domain IVa + V (23.9 kDa) fragments described earlier (40,41). Since we were subsequently able to purify τ, free of contaminating fragments, by overexpression directed by pJC491 in the *ompT* strain BL21(λDE3)*recA*, these data extend earlier observations (57) by showing that τ is sensitive to cleavage by OmpT protease at or near both the Domain III–IVa and Domain IVa–V boundaries (i.e. after Lys429 and Lys498, respectively).

Overproduction and purification of τ_C16 (Domain V)

The part of *dnaX* that encodes τ_C16 (codons 499–643) was amplified by PCR and inserted into a phage T7-promoter vector to generate plasmid pSH1062. However, repeated attempts to introduce pSH1062 into strain BL21(λDE3) *recA* failed to give viable colonies, suggesting that expression of the gene that encodes τ_C16 is, even in a small amounts, lethal to *E. coli*, presumably due to sequestration of α by τ_C16 and consequent inhibition of its binding to native τ. This lethality was exploited in work described later to isolate mutants of τ_C16 that are defective in binding to α. To obtain a strain that overproduces τ_C16, its coding region was subcloned into a phage λ-promoter vector to yield plasmid pSJ1064. Use of more tightly controlled expression from λ-promoters enabled production of the τ_C16 domain in good amounts; we had previously used a similar strategy for the production of the helicase-interaction domain of DnaG primase, which is similarly lethal to *E. coli* (58).

Following overproduction in strain AN1459 containing pSJ1064, τ_C16 was first purified in a yield of 13 mg/l of cell culture (Supplementary Data, method A). Its structure was initially examined by NMR measurements using a 0.45 mM solution at pH 7.0 and 30°C. Under these conditions, the protein underwent stepwise proteolysis to yield a final species with a molecular weight of ∼14 000, as assessed by SDS-PAGE. This fragment remained stable for longer than a day of NMR measurements, and the final TOCSY spectrum suggested the 14-kDa product to be a well-structured protein suitable for structure determination by NMR (43). A second sample of similarly purified τ_C16 that had undergone partial proteolysis was chromatographed on a Mono-Q column, which separated a series of fragments (Supplementary Figure S6). The smallest of these had a molecular weight of 14 334.4 ± 1.9, determined by ESI-MS, which indicated it most likely comprised residues 499–625 (14 334.2 Da, including the N-terminal Met introduced in cloning) or perhaps, 517–642 (14 348.1 Da) of τ. PCR-generated gene fragments encoding each of these fragments were inserted into the λ-promoter vector pND706 (48). Although both of these plasmids directed overproduction of an ∼14-kDa protein (not shown), only the product corresponding to residues 499–625 of τ (encoded in plasmid pSJ1308) was in the soluble fraction following cell lysis. We concluded this corresponded to the stable proteolysis product of τ_C16, and called the protein τ_C14. It lacks the last 18 residues from the C-terminus of τ_C16 (or τ). We found subsequently that more stable samples of τ_C16 could be prepared by a modified procedure (method B, see Materials and methods section) which used strain BL21(λDE3)*recA*/pSJ1064 for expression, and included treatment of the extract with protease inhibitors. The τ_C14 domain was similarly purified in good yield using strain BL21(λDE3)*recA*/pSJ1308.

In contrast to the T7-promoter plasmid encoding τ_C16 (i.e. pSH1062), a similar plasmid (pKO1296) containing the gene for τ_C14 (44) could easily be introduced into and maintained in strain BL21(λDE3)*recA*, indicating that removal of the last 18 residues of τ_C16 suppressed its lethal phenotype. This provided the first indication that these residues might be involved in the α–τ interaction.

The C-terminal 18 residues of τ are required for interaction with α

We next used analytical gel filtration on a column of Sephacryl S100 HR to confirm that τ_C16, but not τ_C14, could form an isolable complex with α. The α subunit alone or in complex with τ_C16 elutes in the void volume, while excess of the small τ fragments are well resolved in the included volume (Figure 2A and B). The results clearly showed that while τ_C16 forms a stable isolable complex with α, τ_C14 did not (Figure S7A), indicating that the last 18 amino acids of τ are required for the α–τ interaction.

In published work, we have shown that this region of τ_C16 is unstructured in solution (44); we presume it adopts an ordered structure when it interacts with α. Next we used SPR experiments with a BIACORE instrument to determine the interaction strength contributed by this 18-residue segment.

For comparison with previously reported experiments (40), we first prepared a N-terminally biotinylated version of τ_C16 (bio-τ_C16), bound it to streptavidin on the surface of a Biacore SA chip, and analyzed its interaction with α by SPR. Because of the slow diffusion of the 130-kDa α subunit through the dextran layer of the SPR chip, quantification of dissociation constants (K_D) required low loadings of bio-τ_C16, use of a high flow rate (30 μl/min) and correction of the data for mass transfer limitations as implemented in the Biacore software package. Sensorgrams recorded at a range of concentrations of α fit well globally to a 1:1 Langmuir binding model (with mass transfer) to yield a value of $K_D(\alpha$–$\tau_C16)$ of
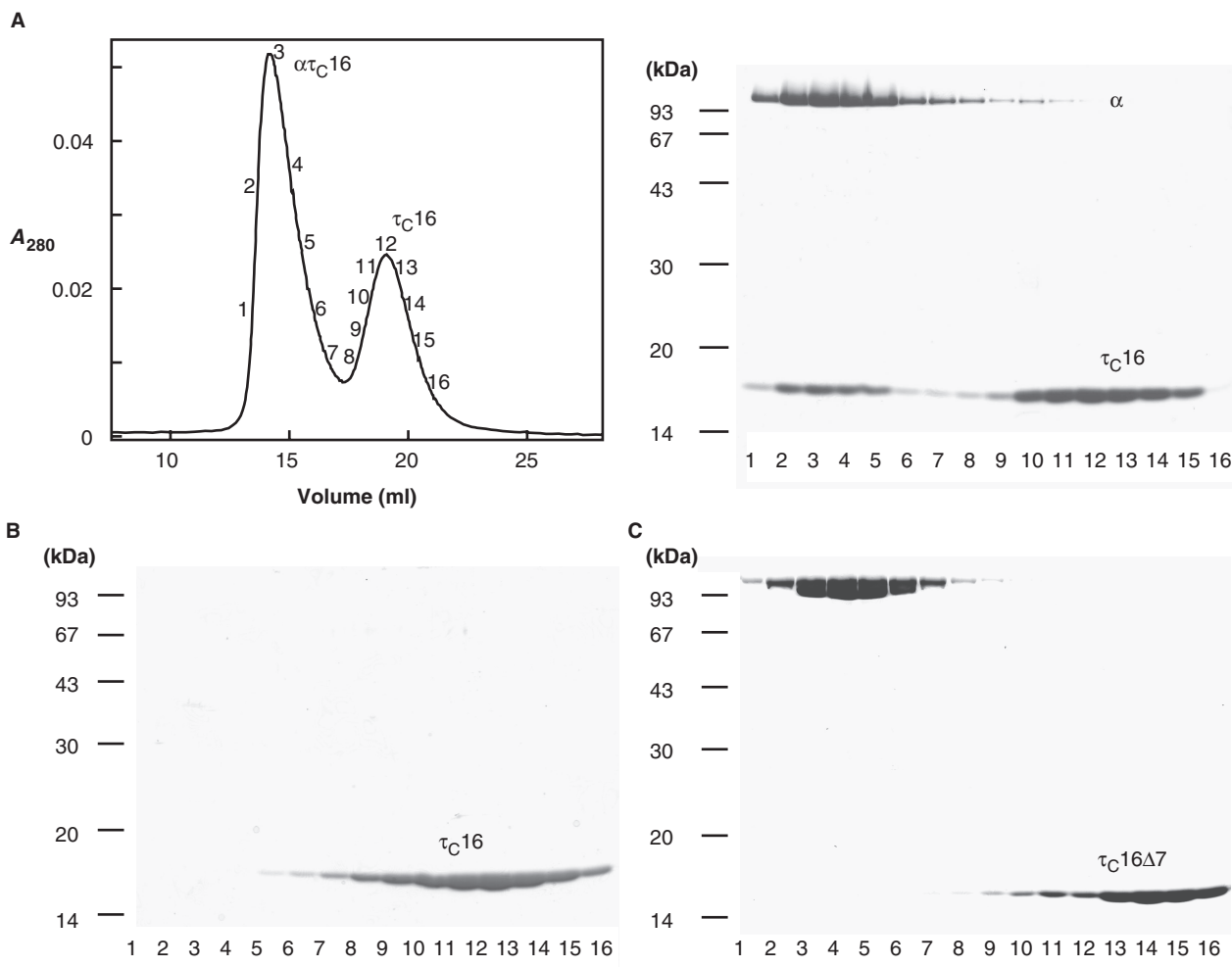

Figure 2. Deletion mutant $\tau_C16\Delta7$ does not form a stable complex with α. (**A**) Formation of a stable complex between *E. coli* α and τ_C16. The α–τ_C16 complex was resolved on a Sephacryl S-100 column. Samples from fractions (indicated by numbers in the left panel) were analyzed by 15% SDS-PAGE. The numbering of lanes corresponds to the numbering shown in the gel filtration profiles. (**B**) Under the same conditions, τ_C16 elutes as a single peak in the included volume. (**C**) Investigation of the interaction of α and $\tau_C16\Delta7$ by gel filtration. Under the same conditions as used to study the α–τ_C16 complex, no stable interaction between α and $\tau_C16\Delta7$ was detected.
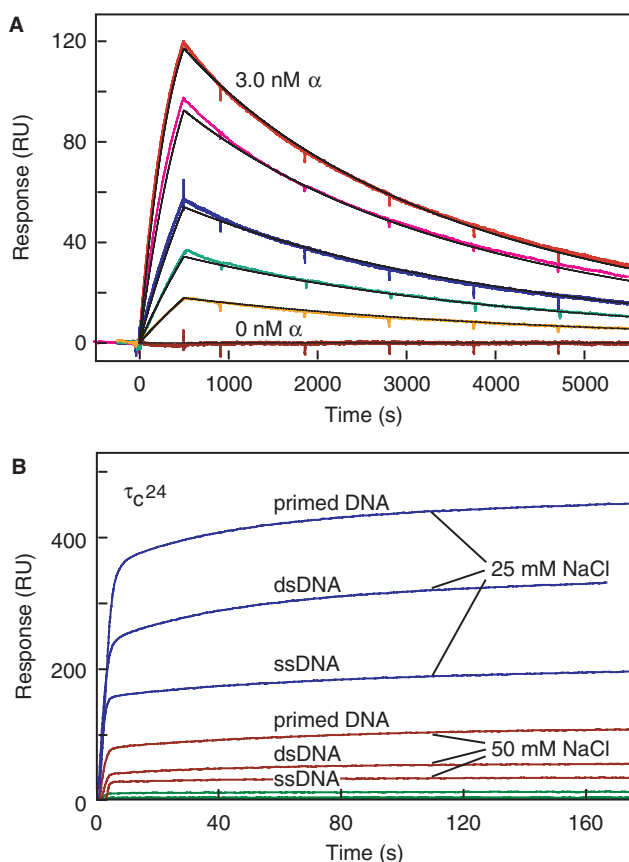
Figure 3. SPR measurements of the $\alpha - \tau_C16$ and DNA–τ_C24 interactions. (**A**) Solutions of decreasing concentrations of α (3.0, 2.0, 1.0, 0.6, 0.3 nM and blank; from top to bottom) were tested for interaction with immobilized bio-τ_C16. Curves, shown in colours, were fitted globally to a 1:1 Langmuir model with mass transfer using BIAevaluation 3.1 software. Sensorgrams were simulated (black curves) using the same model and the fitted parameters $k_a = (1.52 \pm 0.01) \times 10^6\,\mathrm{M^{-1}\,s^{-1}}$, $k_d = (4.02 \pm 0.01) \times 10^{-4}\,\mathrm{s^{-1}}$, k_t (mass transfer coefficient) = $2.98 \times 10^8\,\mathrm{RU\,M^{-1}\,s^{-1}}$, yielding $K_D(\alpha-\text{bio-}\tau_C16) = 265 \pm 2\,\mathrm{pM}$. Measurements were carried out in SPR buffer at 20°C. (**B**) Salt dependence of binding of τ_C24 (1 μM) to ss, ds and primer-template DNA, studied by SPR at 20°C, as described in the Materials and methods section. Binding was monitored in 10 mM Na.HEPES buffer, pH 7.4, 0.005% P20, containing 75 mM NaCl (green sensorgrams), 50 mM NaCl (red) or 25 mM NaCl (blue). Under all conditions studied, the dissociation phase was complete within seconds, indicating that the interaction is rapidly reversible (not shown). Similar responses were seen with τ_C22, while no binding was detected in any condition with τ_C16.

$(2.65 \pm 0.02) \times 10^{-10}\,\mathrm{M}$ (Figure 3A). This value was about two orders of magnitude higher than measured previously (40), largely because of a higher dissociation rate under our experimental conditions. Although the source of this difference has not yet been determined, the data clearly confirm that Domain V of τ interacts strongly with α.

For comparison, biotinylated τ_C14 (bio-τ_C14) was prepared and similarly immobilized to assess its interaction with α; no binding was detected even when 1 μM α was injected (not shown), indicating $K_D(\alpha-\tau_C14) > 10^{-6}\,\mathrm{M}$. These data confirm that the unstructured C-terminal 18-residue segment of τ present in τ_C16 but not τ_C14 is essential for interaction with α.

α interacts weakly with the separated C-terminal region of τ

Next we sought to determine if all interactions of τ_C16 with α are mediated by the unstructured C-terminal segment by SPR measurements of the interactions of α with synthetic 20- and 32-mer peptides corresponding to this portion of τ (designated $\tau_C$pep20 and $\tau_C$pep32, respectively; Figure 1A). N-terminally biotinylated $\tau_C$pep20 and $\tau_C$pep32 were first immobilized directly onto Biacore SA chips. However, we were unable to detect interaction of α (at 1 μM) with these surfaces (not shown). The most likely explanation for this behavior is that peptides positioned close to the chip surface are not accessible to α.

As an alternative method to evaluate α–peptide interactions, we used a competitive binding assay, where 1 nM α was mixed with various concentrations of the unbiotinylated competitor peptide [*I*], and then passed over bio-τ_C16 immobilized on the surface of the SPR chip. An apparent value of K_D (i.e. $K_{D,\text{app}}$) was then measured at each concentration of competitor using the Biacore software, with correction for mass transfer. Linear plots of $K_{D,\text{app}}$ versus [*I*] according to Equation (1) (see right panels in Supplementary Figure S8) were then used to determine values of $K_D(\alpha-I)$ as described in the Materials and methods section. To validate this method, we examined competition by τ_C16 itself, and obtained a value of $K_D(\alpha-\tau_C16)$ of $(1.3 \pm 0.4) \times 10^{-10}\,\mathrm{M}$ (Figure S8A). That this value is close to that determined above by study of the interaction of α directly with immobilized bio-τ_C16 indicates that the interaction is a simple one, that extension of τ_C16 by an N-terminal biotin-tag does not seriously affect its interaction with α, and that measurements of $K_D(\alpha-I)$ obtained by this competition assay can be used reliably to compare strengths of interactions between α and fragments or mutant derivatives of τ_C16.

$K_D(\alpha-\tau_C\text{pep32})$ measured in this way was 3.8 ± 0.2 μM (Figure S8B). That this value is 30 000-fold greater than $K_D(\alpha-\tau_C16)$ suggests that residues located in the folded (τ_C14) core contribute to the binding of τ_C16 to α. Assuming the free energies of binding ($\Delta G°$) to the two sites are additive, then binding to residues in τ_C14 is calculated to contribute about 5.7 kcal/mol, corresponding to $K_D \sim 60$ μM, which is a value too high to be measured reliably by SPR. This is in accord with the lack of detectable interaction between bio-τ_C14 and α.

Residues at the extreme C-terminus of τ are required for binding to α

We used additional approaches to further probe interactions of the C-terminus of τ with α. Guided by sequence alignments that show several amino acids in this region to be highly conserved (see Figure 1B and ref. 43), perhaps because they interact with a similarly conserved region of α, we next deleted residues from the C-terminus of τ_C16, and measured their affinity for α both by gel filtration (Figures 2C and S7B) and in the competitive SPR assay (Figure S8C and Table 1). Deletion of either 7 (in $\tau_C16\Delta7$) or 11 residues ($\tau_C16\Delta11$) from the C-terminus of τ_C16 resulted in soluble proteins that could be purified readily;

Table 1. Equilibrium constants K_D and differences in free energies $\triangle\triangle G^\circ$ for interactions of α with τ_C16 and mutant derivatives

Interaction	K_D (M)	$\triangle\triangle G^\circ$ (kcal/mol)
Direct SPR assay:		
α–bio-τ_C16	$(2.64 \pm 0.02) \times 10^{-10}$	
Competitive SPR assay:		
α–τ_C16	$(1.3 \pm 0.4) \times 10^{-10}$	(0.0)
α–$\tau_C$pep32	$(3.8 \pm 0.2) \times 10^{-6}$	5.9 ± 0.2
α–$\tau_C16\triangle7$	$(6.7 \pm 1.8) \times 10^{-6}$	6.3 ± 0.4
α–$\tau_C16\triangle11$	$>10^{-5}$	>6.5
α–τ_C14 ($\tau_C16\triangle18$)	$>10^{-5}$	>6.5
α–$\tau_C16[S617P]$	$(1.6 \pm 0.6) \times 10^{-7}$	4.1 ± 0.4
α–$\tau_C16[I618T]$	$(9.8 \pm 1.0) \times 10^{-10}$	1.1 ± 0.3
α–$\tau_C16[L627P]$	$(1.5 \pm 0.4) \times 10^{-6}$	5.4 ± 0.4
α–$\tau_C16[F631I]$	$(9.9 \pm 2.1) \times 10^{-8}$	3.8 ± 0.3
α–$\tau_C16[L635P]$	$(2.4 \pm 0.2) \times 10^{-9}$	1.7 ± 0.3
α–$\tau_C16[D636G]$	$(3.8 \pm 0.5) \times 10^{-9}$	1.9 ± 0.3

removal of these amino acids does not appear to affect folding of the isolated domain, consistent with NMR studies that showed the lack of defined structure for this region (44). Since a T7-promoter vector that contained the gene encoding $\tau_C16\Delta11$ could be easily maintained in strain BL21(λDE3)*recA*, its expression was not toxic to *E. coli*. However, the corresponding T7 vector encoding the $\tau_C16\Delta7$ mutant could not be stably maintained, so a λ-promoter vector was again used to prepare this mutant protein. This suggested that deletion of seven residues may not be sufficient to completely prevent interaction of τ Domain V with α; this was examined further by gel filtration and competitive SPR experiments.

Neither $\tau_C16\Delta7$ nor $\Delta11$ formed a complex with α sufficiently stable to enable its isolation by gel filtration (Figures 2C and S7C). However, weak interaction with $\tau_C16\Delta7$ was detected by SPR experiments; deletion of the 7 C-terminal residues raised the measured K_D to $6.7 \pm 1.8 \, \mu\text{M}$ (Table 1), suggesting that these residues contribute $\sim6.3 \, \text{kcal/mol}$ to ΔG°. However, deletion of four further residues (in $\tau_C16\Delta11$) including the conserved residues Ala633 and Leu635 raised K_D further (to $>10 \, \mu\text{M}$), such that interaction with α could no longer be detected.

Targeted mutagenesis of the C-terminal region of τ_C16

Finally, to further probe the α–τ interaction, we carried out a targeted mutagenesis experiment on the C-terminal region of τ_C16. The gene in the T7-promoter plasmid pSH1062 contains a unique BssHII site situated 28 codons before the stop codon, and a single EcoRI site is placed immediately after the gene. A series of error-prone PCRs were used to target mutations to this region, and the product fragment was digested with BssHII and EcoRI. Separate libraries of mutant gene fragments obtained with different $\text{Mn}^{2+}:\text{Mg}^{2+}$ ratios in the PCRs were then inserted between the corresponding sites in pSH1062, and then transformed into strain BL21(λDE3)*recA*. Nucleotide sequence determination revealed that plasmids in 24 randomly selected survivors that yielded interpretable sequences all carried mutations in the targeted

region. The mutations included: (a) Nine single point mutations, resulting in mutant proteins L627P (three isolates), L635P (two isolates), S617P, I618T, F631I and D636G. The two mutants isolated more than once were derived from separate libraries. (b) Nine double point mutations, giving changes I618N/R628C, A620V/D632V, D621G/F630S, N622I/F631S, I624N/F630L, T626A/I640F, L627P/L635Q, D636N/R641S and P642S plus a TGA to AGA mutation at the stop codon. (c) Five frameshift mutations (three deletions and two insertions); and (d) A single alteration of the stop codon (TGA codon changed to CGA); stop-codon mutations result in extension of the protein by 17 additional residues at the C-terminus.

Of the six single point mutations that suppressed the lethality of the gene encoding τ_C16, four were in codons for conserved hydrophobic residues (Figure 1B) and two were in neighboring residues. To examine the roles, if any, of these amino acids in the τ_C16–α interaction, these six proteins were isolated. All were soluble when overproduced in *E. coli* BL21(λDE3)/pLysS, and could be isolated in good yield.

Contributions of residues in the C-terminus of τ to α binding

Interactions between the mutant proteins and α were first assessed by gel filtration, as before. The results (Supplementary Figure S9) suggest that no single point mutation disturbed the interaction as much as the $\tau_C16\Delta7$ and $\Delta11$ truncations. Nevertheless, it is clear for all of them that interaction with α was impaired, and this was most obvious for mutant $\tau_C16[L627P]$. The relative strength of the interactions between the mutant proteins and α was then quantified more reliably using the SPR competition assay (Table 1 and Supplementary Figures S10 and S11).

The I618T, L635P and D636G mutations had rather modest effects on binding (changes in K_D of <30-fold, $\Delta\Delta G^\circ <1.9 \, \text{kcal/mol}$) and, as seen in the gel filtration data, the largest effect was with the L627P mutation ($5.4 \, \text{kcal/mol}$). The S617P and F631I mutations had intermediate effects ($\Delta\Delta G^\circ$ of $\sim4 \, \text{kcal/mol}$). Thus, although the data cannot determine that the sole reason for the lethal phenotype of modest production of τ_C16 is its interaction with α (as opposed to DNA or another replisomal component), all of the isolated mutant proteins had defects in α binding. This confirms that the unstructured C-terminal region of τ_C16 carries many determinants for interaction with α, and provides clues to the structure of the complex, as discussed further below.

Domain IVa of τ_C24 (and τ_C22) is responsible for its interaction with DNA

The τ_C24 protein (Domains IVa + V) has previously been shown to bind to primed DNA (38), and interaction between the C-terminal (20-mer) peptide from α and τ_C24 was proposed to be modulated by DNA structure (39). Accordingly, we used three different constructs, τ_C24, τ_C22 and τ_C16 in SPR experiments with ss, ds and primer-template DNA to map the region responsible for DNA

binding. Compared to τ_C24, τ_C22 lacks the 18 residues from the C-terminus that are involved in interaction with α (Figure 1A). Like τ_C14, τ_C22 could be overproduced in strain BL21(λDE3)/pLysS/pKO1297, where its synthesis is under control of a phage T7-promoter.

Surprisingly, we were unable to detect interaction between any of these protein constructs and any of the immobilized DNA structures in a buffer containing 150 mM NaCl. Further experiments using the same buffer containing 10 mM Mg(OAc)$_2$ in the presence or absence of 1 mM ATP or AMP-PNP also yielded no evidence of interaction, and reduction of [NaCl] to 100 mM yielded similar negative results.

However, reduction of the NaCl concentration to 75 mM yielded detectable interaction between τ_C24 (and τ_C22, not shown) and all DNA structures, while further progressive decrease in [NaCl] to 50 and 25 mM led to stronger, non-stoichiometric binding (Figure 3B). The data show that as more nucleotides are immobilized on the surface (more negative charges), the stronger the response, suggesting that the interaction between τ_C24 (or τ_C22) and DNA is largely electrostatic. Otherwise, there appeared to be no significant preference for binding to primer-template DNA than to ss or dsDNA.

In contrast, Domain V alone (in τ_C16) was observed not to bind to any of the DNA structures even at the lowest salt concentrations. The data therefore show that the 69 residues comprising Domain IVa of τ_C24 (and τ_C22) contain all determinants for DNA binding, and the strong salt dependence and lack of apparent specificity is likely due to the high net positive charge of this region coupled with its lack of defined tertiary structure (43).

DISCUSSION

Structural aspects of the α–τ_C16 interaction

This report shows the important role the extreme C-terminal region of the τ subunit of Pol III HE plays in its interaction with the α polymerase subunit. In the accompanying article (43), we describe the solution structure of the folded core of the C-terminal Domain V of τ (i.e. τ_C14) determined by NMR spectroscopy. The region between residues Pro507 and Ser617 of Domain V is structured; the last eight residues of τ_C14 were found to be mobile (43) and the following 18 residues present in τ_C16 beyond the end of τ_C14 (Figure 1A) are also unstructured and show no evidence of association with the folded core of Domain V (44). Nevertheless, that a peptide comprising just the last 32 residues of τ ($\tau_C$pep32) and including all of this unstructured region does not associate as strongly with α as τ_C16 (Table 1) suggests that some residues in the folded region of Domain V directly or indirectly contribute to the interaction with α.

Sequence alignment reveals that certain residues in the C-terminal region of τ_C16 are highly conserved (Figure 1B, and ref. 43), and our mutagenesis data suggest that these conserved residues are important for interaction with α. This interaction thus provides yet another example of how an intrinsically unstructured region of a protein can fold into a defined structure when it interacts with

its binding partner (59,60). Although this region contains many polar and charged amino acids, most of the conserved residues are non-polar and are spaced in such a way that suggests the folded structure of this part of τ in the α–τ complex comprises two amphipathic α-helices.

Secondary structure predictions suggest an extension of the C-terminal helix 6 of τ_C14 (43) which includes the conserved residues Ala614 and Ile618 (Figure 1B). We studied two single point mutants from this region, S617P and I618T. While the I618T mutation had only a modest effect on α binding ($\Delta\Delta G° = 1.1$ kcal/mol), the effect of the S617P mutation was larger (4.1 kcal/mol). Since the proline substitution at Ser617 would be expected to disrupt an α-helix, this is consistent with the interactions with α occurring via an incipient helix.

Structure prediction further suggests a β-turn involving Asp621–Asn623 followed by a helix from Ile624 to Phe631 with high propensity, or to Leu635 with lower propensity. This suggests that a second longer helix extending to Leu635 might be formed upon interaction with α. Consistent with this is the fact that the conserved residue mutant L635P located at the end of this hypothetical helix (Figure 1B) still interacts relatively strongly with α ($\Delta\Delta G° = 1.7$ kcal/mol) while the L627P mutation within it has the largest effect of those examined (5.4 kcal/mol). Taken together, these data suggest that a helix–loop–helix structure is induced on interaction with α, with the extended helix 6 serving as a scaffold that links the globular domain of τ_C14 and α. Whether the globular domain itself establishes contacts with α in either of the two proposed α–τ binding modes (38) remains to be shown. NMR studies of the interaction of $\tau_C16\Delta11$ with α yielded a dissociation constant of ~0.9 mM (61), indicating that additional binding residues are located within the structured portion near the end of helix 6. Two structures of α, from different bacteria, have recently been reported (62,63). Since τ_C16 is expected to be bound close to the C-terminus of α due to its close proximity to the β_2 sliding clamp that binds in this region, one could speculate that the incipient additional helix 7 binds in a C-terminal cleft in α.

Of the remaining mutants examined, the D636G change had a modest effect on α binding ($\Delta\Delta G° = 1.9$ kcal/mol) while F631I resulted in a much weaker interaction (3.8 kcal/mol). Consistent with these data, Asp636 is not highly conserved while Phe631 is, and since isoleucine is a helix-breaking residue, the F631I mutation would be expected to disrupt the proposed helical structure in this region.

Study of the single point mutants (Table 1) revealed that no single amino acid change is sufficient to completely disrupt the α–τ interaction, presumably because the interactions at the interface involve a series of residues located in an otherwise flexible segment of τ. Finally, we note that although no single point mutation was identified in the last seven amino acids of τ_C16, complete removal of this segment (in $\tau_C16\Delta7$; Figure 1B) had a large effect of binding of α ($\Delta\Delta G° = 6.3$ kcal/mol). There must therefore be further contacts with this region in the complex with α. Indeed, extension of this region in

full-length τ by fusion of a biotin-tag sequence also disrupts the interaction (40). NMR studies (43) similarly suggest that all determinants in τ_C16 required for binding to α likely reside towards the end of helix 6 and in the following unstructured 26-residue segment that we propose to become structured on α binding.

Structural aspects of the α–τ interaction

The DnaB helicase interacts with Domain IV of τ, a region that immediately precedes helix 1 in the structure of τ_C14 (Figure 1) and an α subunit interacts with the extension of helix 6 of the τ_C14 domain in each of the two τ subunits in the clamp loader complex in the replisome (4–7). This places restraints on the α–DnaB and α–α distances that need to be considered once there is sufficient information to build models of the structure of the replisome in its various functional states.

The DNA-binding properties of τ_C24 place further structural restraints. The τ subunit is known to house a DNA-sensing function that leads to active detachment of α from the β_2 clamp when the last nucleotide of an Okazaki fragment on the lagging strand is incorporated, resulting in the processivity switch (6,7,38). Further, it was shown that Domain IVa + V of τ (τ_C24) senses a primed template structure, resulting in lowering of its affinity for the C-terminal region of α (39). Although we have shown that the highly basic Domain IVa region of τ_C24 interacts with DNA, we have been unable to confirm under our experimental conditions that it binds primer-template DNA with high affinity or significant specificity. Domain IV has previously been shown not to contribute directly to α binding (40). Considering the close proximity of the N- and C-terminal helices in the globular fold of Domain V (43), the DNA-binding Domain IV may readily interact with the α-binding site of τ to regulate the $\alpha-\beta_2$ interaction when Domain IV senses changes in DNA structure. Possibly, the affinity with the lagging strand is enhanced through interaction between Domains IV and/or V in neighboring τ subunits in the clamp loader complex. This would place rather severe geometric constraints on the positions of the two Pol III cores and DnaB in the replisome.

SUPPLEMENTARY DATA

Supplementary data is available at NAR Online.

ACKNOWLEDGEMENTS

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