SUPPLEMENTARY DATA:

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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SUPPLEMENTARY MATERIALS:

Oligonucleotide primers

408: 5'-AGATATACATATGAAAGATGAAACCGGCAGCCGCTACCCG-3' (39-mer, NdeI site indicated);
409: 5'-AGATATACATATGAAAGGCGCTGAAACATGAAAAACGCG-3' (40-mer; NdeI site indicated);
438: 5'-TCGAATTCTCAATGGAGGCGGATCTTTCTTCATCCAGCTCCG-3' (44-mer; EcoRI site indicated);
515: 5'-CCTTTCGGGGCTTTGTAAGCAG-3' (21-mer);
806: 5'-GATATACATATGAAAGGCTGCGCAACCGCTGTCGTGGTAATACGCTGC-3' (62-mer, NdeI site indicated);
853: 5'-AAGAATTTCTGAAATATTATTATCGCAATAATGGACTC-3' (41-mer; EcoRI site indicated);
855: 5'-AAAAACATATGGAACGCTGGAAACATGAAAAACGC-3' (39-mer; NdeI site indicated);
907: 5'-TAATTGGCTGTCTGAAACGACATCTTTGAGCTCAGAAAATCGAATGGCAGCAGAACCAT-3' (60-mer; NdeI site indicated);
908: 5'-CGCGTTCATATGTTTCTGAGCTTTTTCTGAGCTTCTGCAAGATGTCGTTCAAGACAGCATC-3' (62-mer; NdeI site indicated);
987: 5'-AAAAACATATGAAAGGTCGTGACGCACTGGCTGCAACCGCTGCTGTCGATGTC-3' (65-mer, NdeI site indicated);
998: 5'-TTTTTTTTTTCATATGAAAGGATAAAGCCTGGCC-3' (31-mer; NdeI site indicated);
999: 5'-TTTGAATTCATTATATTTTCTGCACTACGC-3' (28-mer; EcoRI site indicated);
1078: 5’-AATTCTTAATCCAGCTCCGCATCGAAGAACCGACGCAGGGTCTGAA TATTTATCCGCAATAATGGACATCG-3’ (72-mer);
1079: 5’-CGCGCGAGTCCATTATGCGGATAATAATTTAGGCCTGCCTGCGGT TCTTCGATTAAG-3’ (60-mer);
1080: 5’-AATTCTTAATCGAAGAACCGACGCAGGGTCTGAATATTATTATCCG CAATAATGGACATCG-3’ (60-mer);
1083: 5’-CGCGCGAGTCCATTATTGCGGATAATAATATTCAGACCCTGCGTCG GTTCTTCGATTAAG-3’ (72-mer).

Buffers

Buffers were: lysis buffer (50 mM Tris.HCl, pH 7.6, 1 mM EDTA, 1 mM dithiothreitol, 20 mM spermidine); buffer A (50 mM Tris.HCl, pH 7.6, 1 mM EDTA, 1 mM dithiothreitol, 20% v/v glycerol, 150 mM NaCl); buffer C (35 mM Tris.HCl, pH 7.6, 1 mM EDTA, 1 mM dithiothreitol); buffer D (20 mM Tris.HCl, pH 7.6, 0.5 mM EDTA, 1 mM dithiothreitol, 150 mM NaCl); buffer E (20 mM Tris.HCl, pH 7.9, 500 mM NaCl, 2 mM β-mercaptoethanol); buffer F (20 mM Tris.HCl, pH 7.9, 2 mM β-mercaptoethanol); buffer G (30 mM imidazole, pH 7.0, 50 mM NaCl, 5% (v/v) glycerol, 5 mM β-mercaptoethanol); buffer GF (20 mM Tris.HCl, pH 7.6, 0.5 mM EDTA, 1 mM dithiothreitol, 150 mM NaCl); buffer P (20 mM sodium phosphate, pH 6.5, 1 mM EDTA, 1 mM dithiothreitol); biomix buffer (50 mM Tris.HCl, 250 mM bicine, pH 8.3, 50 mM ATP, 50 mM magnesium acetate, 250 μM biotin); loading buffer (300 mM Tris.HCl, pH 7.6, 15% (v/v) glycerol, 0.6% (w/v) bromophenol blue, 50 mM dithiothreitol, 2% (v/v) SDS).

Minimal autoinduction medium for 15N labelling

This medium (S1) was used to prepare uniformly 15N-labeled proteins. It was comprised of: 100 mM NaH2PO4/KHPO4 at pH 6.75, trace salts, 1 mg/l vitamin B1, 1 mM MgSO4, 4 g/l D(+)-glucose, 5 g/l glycerol, 2 g/l α-lactose, and 1 g/l 15NH4Cl (Cambridge Isotope Laboratories).

Construction of plasmids

pJC491: The plasmid pZT3(1G4G), a kind gift from Dr Zenta Tsuchihashi, contains the 1G4G mutant dnaX gene that has been shown to direct production of τ in the complete absence of γ in the E.coli strain BL21(λDE3) (S2). A 1151-bp mutant fragment of the dnaX gene was isolated from pZT3(1G4G) and ligated between NdeI and NheI sites of the λ-promoter dnaX+ plasmid pJC490 (S3) to give pJC491 (Figure S1). Plasmid pJC491 directs overproduction of the full-length τ subunit, but not γ (data not shown).

pSH1062: The plasmid pPT153 (S3) was used as template for PCR amplification of the gene encoding τC16. The PCR product was generated using primer 409, designed to incorporate a start codon before the Lys499 codon of dnaX as part of an Ndel site and primer 438, designed to preserve an EcoRI restriction site just after the wild type TGA stop codon. The PCR product was isolated following digestion with the restriction enzymes EcoRI and Ndel and electrophoretic separation by extraction from an agarose gel (1.5%). The τC16+ fragment (443 bp) was inserted between the same restriction sites
in the vector pETMCSI (S4), to place the gene under transcriptional control of the phage T7 φ10 promoter in plasmid pSH1062.

Figure S1. Construction of the plasmid pJC491, which directs the overproduction of τ in the absence of γ from a mutant dnaX gene. Reproduced from Crowther (S9).

pSJ1064: The plasmid pSH1062 was digested with Ndel and EcoRI. The 443-bp τC16+ fragment was isolated following electrophoretic separation by extraction from an agarose gel (1.5%). The fragment was inserted between the same restriction sites in vector pND706 (S5), to place the gene under the transcriptional control of tandem bacteriophage λ pR and pL promoters in the 4725-bp plasmid pSJ1064 (Figure S2).

pSJ1308: Plasmid pJC491 (Figure S1) was used as template for PCR amplification of the gene that encodes τc14. The PCR product was generated using primer 855, designed to incorporate a start codon before the Lys499 codon of dnaX as part of an Ndel site and primer 853, designed to incorporate an EcoRI restriction site just after a TAA stop codon so that Gln625 of τ is the C-terminal residue. The PCR product was digested with Ndel and EcoRI and isolated following electrophoretic separation by extraction from an agarose gel (1.5%). The τC14+ fragment (389 bp) was inserted between the same restriction sites in the vector pND706 (S5) to give the 4671-bp plasmid pSJ1308 (Figure S3).
Figure S2. Construction of the plasmid pSJ1064, which directs the overproduction of $\tau_{C16}$ from a mutant dnaX gene.

**pSJ1318**: The two oligonucleotide primers 1079 and 1080 were mixed in equimolar amounts, annealed and the resulting self-complementary linker ligated between the BssHII and EcoRI sites in pSH1062 to yield plasmid pSJ1318. This plasmid contains the gene encoding $\tau_{C16\Delta11}$ inserted between Ndel and EcoRI restriction sites in the T7-promoter vector pETMCSI (S4) and directs overproduction of $\tau_{C16\Delta11}$ when induced by IPTG.

**pSJ1319**: The two oligonucleotide primers 1078 and 1083 were mixed in equimolar amounts, annealed and the resulting self-complementary linker ligated between the BssHII and EcoRI sites in pSH1062 to yield plasmid pSJ1319. This plasmid contains the gene encoding $\tau_{C16\Delta7}$ inserted between Ndel and EcoRI restriction sites in pETMCSI (S4).

**pSJ1326**: The plasmid pSJ1319 was cut by Ndel and EcoRI and the 422 bp fragment bearing the gene encoding $\tau_{C16\Delta7}$ isolated following electrophoretic separation and extraction from an agarose gel (1.5%). The fragment was inserted between the same restriction sites in the vector pND706 (S5), to yield the $\lambda$ promoter plasmid pSJ1326, which directs overproduction of $\tau_{C16\Delta7}$ when induced by a temperature shift from 30 to 42°C.
Figure S3. Construction of the plasmid pSJ1308, which directs the overproduction of wild type $\tau_{C14}$ from a fragment of the $dnaX$ gene.

**pSH1063:** Plasmid pPT153 (S3) was used as template for PCR amplification of the gene encoding wild-type $\tau_{C24}$. The PCR product was generated using the primer 408, designed to incorporate a start codon before the Lys430 codon of $dnaX$ as part of a NdeI site and primer 438, designed to incorporate an EcoRI site just after the wild-type TGA stop codon. The PCR product was isolated following digestion with EcoRI and NdeI, and electrophoretic separation and extraction from a 1.5% agarose gel. The wild-type $\tau_{C24}^+$ fragment (650 bp) was inserted between the same restriction sites in the T7 promoter vector pETMCSI (S4) to give plasmid pSH1063. Although pSH1063 could successfully be transformed into *E.coli* BL21(λDE3)recA, the resulting strain did not produce detectable amounts of $\tau_{C24}$ protein. We considered it likely that poor expression was due to poor mRNA translation due to the presence near the start codon of the $\tau_{C24}$ mRNA of the stem-loop structure normally involved in programmed translational frameshifting during $dnaX$ expression. To remove the stem-loop without changing the amino acid sequence of the $\tau_{C24}$ protein, we used new PCR primers to introduce silent mutations into this region.

**pSJ1330:** Plasmid pSH1063 was therefore used as template for PCR amplification of the silent-mutated gene that encodes wild-type $\tau_{C24}$. The PCR product was generated using the primer 806, designed to incorporate a start codon before the Lys430 codon of $dnaX$ as part of an NdeI site and the primer 515, designed to preserve an
EcoRI restriction site after the wild-type TGA stop codon within pSH1063. The PCR product was isolated following digestion with EcoRI and Ndel, and electrophoretic separation and extraction from a 1% agarose gel. The fragment (650 bp) was inserted between the same restriction sites in pND706 (S5) under transcriptional control of bacteriophage λ promoters to give plasmid pSJ1330 (4932 bp). This plasmid directed high-level expression of τC24 protein.

**pSJ1331:** Plasmid pJC491 (Figure S1) was used as template for PCR amplification of the τC22 gene. The PCR product was generated using the primer 987 (note that this primer is similar to primer 806 but it has 8 instead of 6 nucleotides on the 5'-side of the Ndel restriction site to facilitate easier Ndel digestion) and primer 853, designed to incorporate an EcoRI restriction site just after a new TAA stop codon, such that Gln625 of τ is now the C-terminal residue. The PCR product was isolated following electrophoretic separation and extraction from a 1% agarose gel, after being digested with EcoRI and Ndel. The resulting τC22+ fragment (596 bp) was inserted between the same restriction sites in pND706 (S5) to give plasmid pSJ1331 (4878 bp).

**pKO1297:** Plasmid pSJ1331 was cut by Ndel and EcoRI and the 596-bp τC22+ fragment isolated following electrophoretic separation and extraction from a 1% agarose gel. The fragment was inserted between the same restriction sites in pETMCSI (S4) to give the T7 promoter τC22 overproduction vector pKO1297.

**pKO1274:** A biotin-tag encoding sequence MAGLNDIFEAOK\textsuperscript{bio}IEWHEH (ref. S6; #85 peptide sequence underlined) was placed to provide a specific mono-biotinylation site at the N-terminus of downstream proteins of interest. Two oligonucleotide primers 907 and 908 were mixed in equimolar amounts and annealed, and the resulting self-complementary linker ligated between the Ndel and MluI sites in pETMCSI (S4) to give plasmid pKO1274. This operation resulted in elimination of the first Ndel site upstream of the biotin-tag sequence and allowed us to fuse a gene in-frame downstream of the N-terminal 18 amino acid biotin-tag sequence using unique Ndel site, MluI, and EcoRI sites in this order.

**pKO1282:** This plasmid which directs overproduction of bioτC14 was constructed by ligation of the 389 bp Ndel–EcoRI digested PCR fragment amplified from pSJ1308 between the corresponding sites in pKO1274 to give a T7 promoter biotin-tag fused τC14 overexpression vector.

**pKO1283:** This plasmid was constructed by ligation of the 443 bp Ndel–EcoRI fragment from pSJ1064 between the corresponding sites in pKO1274 to give a T7 promoter biotin-tag fused τC16 overexpression vector.

**pKO1284:** This plasmid which directs overproduction of bioτC16 was constructed by ligation of the 537 bp XbaI–EcoRI fragment from pKO1283 between the corresponding sites in pND706 (S5) to give a λ promoter biotin-tag fused τC16 overexpression vector.

**pKO1289:** This biotin-ligase overproducing plasmid was constructed by ligation of the 972 bp birA Ndel–EcoRI digested PCR fragment derived from E. coli AN1459 (S7) genomic DNA using primers 998 and 999 between the corresponding sites in
pETMCSIII (S4) to give a T7 promoter (His)$_6$-tag fused biotin ligase overexpression vector.

**Protein overproduction and purification**

All steps following cell growth were carried out at 4°C. FPLC or Akta Explorer systems (Amersham Pharmacia Biotech) were used for chromatographic steps. Molecular weights of purified proteins were determined by ESI-MS using a VG Quattro II mass spectrometer with samples that had been dialyzed extensively into 0.1% formic acid and 1 mM 2-mercaptoethanol. Calculated molecular weights were from the Expasy server (www.expasy.org).

**Purification of $\tau_{C16}$ (method A):** The *E. coli* strain AN1459/pSJ1064 was grown at 30°C in LB medium containing ampicillin (200 mg/l) to $A_{595} = 1.2$. To induce overproduction, the temperature was increased to 42°C. The 1-liter cultures were shaken for a further period of 2.5 h, then chilled in ice. Cells were harvested by centrifugation (11000 × g; 5 min), frozen in liquid nitrogen and stored at −70°C.

After thawing, cells (10 g from 3 liters of culture) were resuspended in lysis buffer (150 ml) and lysed by being passed twice through a French press (12000 psi). The lysate was clarified by centrifugation (35000 × 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 (35000 × g; 30 min) and dissolved in buffer A+150 mM NaCl (40 ml). The solution was dialyzed against three changes of 2 l of the same buffer, to yield Fraction II.

Fraction II was applied at 1 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 1 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 100 ml of the same buffer, $\tau_{C16}$ was eluted in a linear gradient (350 ml) of 0–350 mM NaCl in buffer A. Fractions containing $\tau_{C16}$ were pooled and dialyzed against three changes of 2 l of buffer A, to yield Fraction IV.

Fraction IV (28 ml) was split on 2 parts, each of which was applied separately at a flow rate of 1 ml/min onto a Mono Q HR 10/10 column (Pharmacia) that had been equilibrated in buffer A. The $\tau_{C16}$ subunit was eluted at about 70 mM NaCl in a linear gradient (45 ml) of 0–250 mM NaCl in buffer A. Fractions containing $\tau_{C16}$ were pooled and dialyzed against three changes of 2 l of buffer B to yield fraction V (20 ml, containing 38 mg of protein). Aliquots were frozen and stored at −70°C.

ESI-MS gave a value of 16525 ± 2 for the molecular weight of $\tau_{C16}$, which may be compared to the calculated value of 16523.68 (assuming that the N-terminal methionine had not been removed). Problems with adventitious proteolysis of $\tau_{C16}$ prepared by this method were overcome by development of the alternative method B (see Materials and Methods). SDS-PAGE analysis of fractions from a purification using method B is shown in Figure S4.
Figure S4. Overproduction and purification of $\tau_{C16}$ (method B). Samples from successive purification steps were analyzed on a 15% SDS-PAGE gel. Lanes are marked I–VI and correspond to fractions described in Materials and Methods: I, whole cells, before induction; II, whole cells, after induction; III, lysate supernatant (Fraction I); IV, ammonium sulfate pellet (Fraction II); V, first DEAE-650M flow through (Fraction III); VI, second DEAE-650M pool (Fraction IV).

Purification of $\tau_{C14}$: The E.coli strain BL21(λDE3)recA/pSJ1308 was grown in 1-liter cultures at 30°C in LB medium containing ampicillin (150 mg/l) to $A_{595} = 1.2$. To induce overproduction, the temperature was increased to 42°C. Cultures were shaken for a further period of 3 h, then chilled in ice. Cells were harvested by centrifugation (11000 $\times$ g; 5 min), frozen in liquid nitrogen and stored at −70°C.

After thawing, cells (16 g from 6 l of culture) were resuspended in lysis buffer (240 ml). PMSF was added to 0.7 mM just before the cells were lysed by being passed twice through a French press (12000 psi). The lysate was clarified by centrifugation (35000 $\times$ 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 (35000 $\times$ g; 30 min) and dissolved in buffer C+150 mM NaCl (45 ml). The solution was dialyzed against three changes of 2 l of the same buffer, to yield Fraction II.

Fraction II was applied at 1 ml/min onto a column (2.5 $\times$ 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 C. The dialysate (Fraction III, 50 ml) was loaded at a flow rate of 1 ml/min onto a column (2.5 $\times$ 16 cm) of the same resin, now equilibrated in buffer C. The column was washed with buffer C until $\tau_{C14}$ eluted between 1.5 and 2.5 column volumes in a relatively sharp peak considering that the purification is based on late elution (with an observed focusing effect). Fractions containing highly purified $\tau_{C14}$ were pooled, concentrated by precipitation with solid ammonium sulfate (0.45 g/ml), redissolved and...
dialyzed against three changes of 2 l of buffer D, to give Fraction IV (8 ml, containing 110 mg of protein). Aliquots were frozen in liquid nitrogen and stored at –70˚C.

ESI-MS gave a value of 14333 ± 1 for the molecular weight of $\tau_{C14}$, which may be compared to the calculated value of 14334.23 (assuming that the N-terminal methionine had not been removed). SDS-PAGE analysis of fractions collected during purification of $\tau_{C14}$ is shown in Figure S5.

**Figure S5.** Overproduction and purification of $\tau_{C14}$. Samples from successive purification steps were analyzed on a 15% SDS-PAGE gel. I, whole cells, before induction; II, whole cells, after induction; III, lysate supernatant (Fraction I); IV, ammonium sulfate pellet (Fraction II); V, first DEAE-650M flow through (Fraction III); VI, second DEAE-650M pool (Fraction IV).

**Purification of bio-$\tau_{C14}$:** *E. coli* strain BL21(λDE3)recA/pKO1294 was grown at 30°C in LB medium containing ampicillin (150 mg/l) and 15 μM biotin to $A_{595} = 1.0$. To induce overproduction, the temperature was increased to 42°C. Cultures were shaken for a further 2.5 h, then chilled in ice. Cells were harvested by centrifugation (11000 × g; 5 min), frozen in liquid nitrogen and stored at –70°C.

After thawing, cells (5 g from 2 l of culture) were resuspended in lysis buffer (75 mL). A proteinase inhibitor cocktail Complete pill was solubilized according to the manufacturer’s prescription and PMSF adjusted to 0.7 mM just prior to the cells being lysed by being passed twice through a French press (12000 psi). The lysate was clarified by centrifugation (35000 × 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 (35000 × g; 30 min) and dissolved in buffer A+150 mM NaCl (40 ml). The solution was dialyzed against three changes of 2 l of the same buffer, to yield Fraction II (35 ml).

Fraction II was applied at 1.5 ml/min onto 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, 55 ml) was loaded at a flow rate of 1.5 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 180 ml of the same buffer, proteins were eluted using a linear gradient (420 ml) of 0–160 mM NaCl in buffer A. Bio-τC16 eluted in two discrete peaks of which the first one (dominant peak A) eluted at about 50 mM NaCl while the second (peak B) eluted at 80 mM NaCl. Fractions from the two peaks containing purified proteins were separately pooled and dialyzed against three changes of 2 l of buffer GF, to give Fractions IVA (30 ml, containing 38 mg of protein) and IVB (22 ml, containing 9 mg of protein). Aliquots were frozen in liquid nitrogen and stored at –70˚C.

The molecular weights of purified proteins determined by ESI-MS from peak A (18544 ± 1) and peak B (18769 ± 1) indicated the following:
(a) The N-terminal methionine had been removed from both samples;
(b) Only the protein in peak B was biotinylated (bio-τC16) while the protein in peak A did not have attached biotin (bio*τC16);
(c) The complete separation of biotinylated and non-biotinylated protein had been achieved.

**Purification of bio*τC14:** E. coli strain BL21(λDE3)recA/pLysS/pKO1282 was grown at 37˚C in LB medium containing ampicillin (200 mg/l), chloramphenicol (33 mg/l) and biotin (15 µM). Upon growth to an A595 of 1.0, IPTG was added to 0.5 mM and the 1-liter cultures shaken at 37˚C for a further 3 h, after which they were chilled in ice. Cells were harvested by centrifugation (11000 × g; 5 min), frozen in liquid nitrogen and stored at –70˚C.

After thawing, cells (5 g from 2 l of culture) were resuspended in lysis buffer (75 ml). PMSF was added to 0.7 mM just prior to the cells being lysed by being passed twice through a French press (12000 psi). The lysate was clarified by centrifugation (35000 × 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 (35000 × g; 30 min) and dissolved in buffer C+150 mM NaCl (35 ml). The solution was dialyzed against three changes of 2 l of the same buffer, to yield Fraction II.

Fraction II was applied at 2 ml/min onto 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 C. The dialysate (Fraction III, 48 ml) was loaded at a flow rate of 2 ml/min onto a column (2.5 × 16 cm) of the same resin, now equilibrated in buffer C. After the column had been washed with 160 ml of the same buffer, bio-τC14 was eluted using a linear gradient (380 ml) of 0–160 mM NaCl in buffer C. It eluted in a single peak at about 25 mM NaCl. Fractions containing highly purified bio-τC14 were pooled and dialyzed against three changes of 2 l of buffer GF, to give Fraction IV (25 ml, containing 70 mg of protein). Aliquots were frozen in liquid nitrogen and stored at –70˚C.

The molecular weight of the final protein determined by ESI-MS (16353 ± 1) compares well to the calculated value of 16353.43 for bio*τC14 indicating two events: the N-terminal methionine had been removed and biotinylation had not taken place. In fact,
no species of molecular weight of 16579.75 that corresponds to bio-τC14 (where biotin has actually been attached to bio*τC14) was observed in the spectrum.

Since we could not incorporate biotin, an overproduction trial using autoinduction medium (S1) in the presence of excessive biotin (1 mM) was carried out according to the following procedure:

E. coli strain BL21(λDE3)/pLysS/pKO1282 was grown at room temperature in autoinduction medium containing ampicillin (100 mg/l), chloramphenicol (33 mg/l) and biotin (1 mM). After 2 days, cells were chilled in ice, harvested by centrifugation (11000 × g; 5 min), frozen in liquid nitrogen and stored at −70°C.

After thawing, 7 g of cells from 1 liter of culture were resuspended in lysis buffer (110 ml) and 160 mg of bio-τC14 purified the same way as described above. However, the ESI-MS measurement again indicated that biotin has not been attached to the protein.

**Purification of biotin ligase (BirA):** The purification of (His)_6-tagged biotin ligase (BirA) was essentially carried out following the procedure of McIver et al. (S8), modified by an additional chromatography step on a column of Q-Sepharose. E. coli strain BL21(λDE3)/pLysS/pKO1298 was grown at room temperature in two 1-liter cultures in LB autoinduction medium (S1) containing ampicillin (200 mg/l), and chloramphenicol (33 mg/l). Protein production was autoinduced over a period of 48 h as described by Studier (S1). After two days (final A595 = 7.7), cells were harvested by centrifugation (6000 × g; 10 min), frozen in liquid nitrogen and stored at −70°C.

After thawing, cells (12.7 g, from 2 l of culture) were resuspended in lysis buffer (190 ml) and lysed by two passages through a French press operated at 12000 psi. The lysate was clarified by centrifugation (35000 × g, 40 min) to yield soluble Fraction I.

Fraction I was applied at 1 ml/min onto a column (2.5 × 6 cm) of Ni-NTA resin (Qiagen) that had been equilibrated in buffer E. After the column has been washed with 50 ml of buffer E+5 mM imidazole (to remove proteins bound by nonspecific interactions), (His)_6-tagged biotin ligase (BirA) was eluted in a linear gradient (150 ml) of 5–1000 mM imidazole in buffer E. Fractions containing BirA were pooled and dialyzed against 3 changes of 2 l of buffer F to yield Fraction II.

Fraction II was applied at a flow rate of 1 ml/min onto a column (2.5 × 7 cm) of Q-Sepharose resin (Pharmacia) that had been equilibrated in buffer F. The column was washed with 50 ml of the same buffer and biotin ligase was eluted in a single peak using a linear gradient (200 ml) of 0–1 M NaCl in buffer F.

Fractions containing highly purified (His)_6-tagged biotin ligase were pooled and dialyzed against three changes of 2 l of buffer G, to give Fraction III (22 ml, containing 74 mg of protein). Aliquots were frozen and stored at −70°C. ESI-MS gave value of 36267 for the molecular weight of (His)_6-tagged biotin ligase, which may be compared to the calculated value of 36266.0 (assuming that the N-terminal methionine had not been removed).

**In vitro biotinylation of bio*τC14:** Since we were unable to biotinylate bio*τC14 in vivo (see above), biotinylation was carried out in vitro. One part of biomix buffer was mixed with one part of substrate solution (170 µM bio*τC14 in buffer GF) and 3 parts of distilled water; biotin ligase was then added to 1.8 µM. The solution was treated at 30°C for 3 h and then dialyzed in 2 l of buffer GF at 4°C.
The molecular weight of the product protein determined by ESI-MS (16580 ± 1) compares well to the calculated value of 16579.8 for bio-τC14 (assuming that the N terminal methionine had been removed) while the absence of a species with molecular weight of 16353.4 (for bio*τC14) from the spectrum indicated that the protein was completely biotinylated.

**Purification of τC16Δ7:** *E. coli* strain BL21(λDE3)recA/pSJ1326 was grown at 30°C in LB medium containing ampicillin (100 mg/l) to $A_{595} = 1.0$. To induce overproduction, the temperature was increased to 42°C. Cultures were shaken for a further 3 h, then chilled in ice. Cells were harvested by centrifugation (11000 × g; 5 min), frozen in liquid nitrogen and stored at −70°C.

After thawing, cells (5.5 g from 3 l of culture) were resuspended in lysis buffer (120 ml). Proteinase inhibitor cocktail Complete pills (2 pills) were added and PMSF adjusted to 0.7 mM just prior to the cells being lysed by being passed twice through a French press (12000 psi). Fifty mg of τC16Δ7 was isolated according to the method B for purification of τC16 (see Materials and Methods). After final dialysis in buffer GF, aliquots were frozen in liquid nitrogen and stored at −70°C.

ESI-MS gave a value of 15698 ± 1 for the molecular weight of τC16Δ7, which may be compared to the calculated value of 15698.75 (assuming that the N-terminal methionine had not been removed).

**Purification of τC16Δ11:** *E. coli* strain BL21(λDE3)recA/pSJ1318 was grown at 37°C in LB medium containing ampicillin (100 mg/l). Upon growth to an $A_{595}$ of 1.0, IPTG was added to 0.5 mM and the 1-liter cultures were shaken at 37°C for a further 3 h, after which they were chilled in ice. Cells were harvested by centrifugation (11000 × g; 5 min), frozen in liquid nitrogen and stored at −70°C.

After thawing, cells (7 g from 2 l of culture) were resuspended in lysis buffer (105 ml). Proteinase inhibitor cocktail Complete pills (two pills) were added and PMSF adjusted to 0.7 mM just prior to the cells being lysed by being passed twice through a French press (12000 psi). Relatively pure τC16Δ11 (30 mg) was isolated according to the method for purification of τC16 (see above) except that buffer A was used instead of buffer C. After final dialysis in buffer GF, aliquots were frozen in liquid nitrogen and stored at −70°C.

ESI-MS gave a value of 15267 ± 1 for the molecular weight of τC16Δ11, which may be compared to the calculated value of 15270.31 (assuming that the N-terminal methionine had not been removed).

**Purification of τC16 single point mutants:** τC16[S120P], τC16[I121T], τC16[L130P], τC16[F134I], τC16[L138P] and τC16[D139G]: *E. coli* strains BL21(DE3)/pLysS containing each of the plasmids pSJ1320–pSJ1325 (see Materials and Methods) were grown at 37°C in LB medium containing ampicillin (100 mg/l) and chloramphenicol (33 mg/l). Upon growth to an $A_{595}$ of 1.0, IPTG was added to 0.5 mM and the 1-liter cultures were shaken at 37°C for a further period of 3 h, after which they were chilled in ice. Cells were harvested by centrifugation (11000 × g; 5 min), frozen in liquid nitrogen and stored at −80°C.

After thawing, cells (3.5 to 5 g from 2 l of culture) were resuspended in lysis buffer (75 ml). Proteinase inhibitor cocktail Complete pills (1.5 pills) and PMSF (0.7
mM) were added just prior to the cells being lysed with a French press operated at 12000 psi. Between 35–50 mg of each of the six pure \(\tau_{C16}\) single point mutant proteins were isolated according to the method B for purification of \(\tau_{C16}\) (Materials and Methods). The only exception occurred while purifying \(\tau_{C16}[D139G]\); fractions that eluted mostly in the late flow through (instead of in the NaCl gradient) during chromatography on Toyopearl DEAE-650M anion-exchange resin were pooled and then concentrated by addition of solid ammonium sulfate (0.45 g/ml). Finally, each of the solutions of mutant proteins was dialyzed in buffer GF; aliquots were frozen in liquid nitrogen and stored at –70°C.

ESI-MS gave the following values for the molecular weights of \(\tau_{C16}\) mutant proteins: \(16533.3 \pm 0.4\) for \(\tau_{C16}[S120P]\) (calculated value 16533.7); \(16510.6 \pm 0.2\) for \(\tau_{C16}[I121T]\) (16511.6); \(16507.2 \pm 0.5\) for \(\tau_{C16}[L130P]\) (16507.6); \(16489.5 \pm 0.3\) for \(\tau_{C16}[F134I]\) (16489.7); \(16507.8 \pm 0.7\) for \(\tau_{C16}[L138P]\) (16507.6) and \(16466.5 \pm 3.2\) for \(\tau_{C16}[D139G]\) (16465.7).

**Overproduction and purification of \(\tau_{C24}\):** E. coli strain BL21(\(\lambda\)DE3)recA/pSJ1330 was grown at 30°C in LB medium containing ampicillin (100 mg/l) to \(A_{595} = 1.0\). To induce overproduction, the temperature was increased to 42°C. Cultures were shaken for a further 2.5 h, then chilled in ice. Cells were harvested by centrifugation (11000 \(\times\) g; 5 min), frozen in liquid nitrogen and stored at –80°C.

After thawing, cells (12.5 g from 6 liters of culture) were resuspended in lysis buffer (190 ml). The cells were lysed by being passed twice through a French press (12000 psi). The lysate was clarified by centrifugation (35000 \(\times\) g; 30 min) to yield the soluble Fraction I. Proteins that were precipitated from Fraction I by addition of solid ammonium sulfate (0.4 g/ml) and stirring for 60 min, were collected by centrifugation (35000 \(\times\) g; 30 min) and dissolved in buffer A+150 mM NaCl (40 ml). The solution was dialyzed against three changes of 2 l of the same buffer, to yield Fraction II. Fraction II was applied at 1 ml/min to a column (2.5 \(\times\) 16 cm) of Toyopearl DEAE-650M resin that had been equilibrated in buffer A+150 mM NaCl. Fractions containing proteins that did not bind to the column were pooled and dialyzed against three changes of 2 l of buffer A.

The dialysate (Fraction III, 50 ml) was loaded at a flow rate of 1 ml/min onto a column (2.5 \(\times\) 16 cm) of the same resin, now equilibrated in buffer A. The column was washed with buffer A until \(\tau_{C24}\) eluted between 1 and 1.5 column volumes in a sharp peak. Fractions containing \(\tau_{C24}\) were pooled and dialyzed against three changes of 2 liters of buffer P, to give Fraction IV (40 ml). Fraction IV was applied at 0.5 ml/min onto a column (2.5 \(\times\) 12 cm) of phosphocellulose (Whatman P11) resin that had been equilibrated in the same buffer. \(\tau_{C24}\) was eluted using a linear gradient (300 ml) of 0–550 mM NaCl in buffer P. It eluted in a single peak at about 210 mM NaCl. Fractions containing purified \(\tau_{C24}\) were pooled and dialyzed against three changes of 2 l of buffer GF, to give Fraction V (30 ml, containing 33 mg of protein). Aliquots were frozen in liquid nitrogen and stored at –80°C.

ESI-MS gave a value of 24004 ± 1 for the molecular weight of \(\tau_{C24}\), which may be compared to the calculated value of 23999 (assuming that the N-terminal methionine had not been removed).

**Overproduction and purification of \(\tau_{C22}\):** A sample of uniformly \(^{15}\text{N}\)-labeled \(\tau_{C22}\) was prepared from cells of strain BL21(\(\lambda\)DE3)/pLysS/pKO1297 grown in 2 liters of
minimal autoinduction medium for $^{15}$N labeling containing ampicillin (150 mg/l) and chloramphenicol (35 mg/l). Cultures were shaken at room temperature for 72 h (final $A_{595} = 3.0$), harvested by centrifugation (11000 × g; 5 min), frozen in liquid nitrogen and stored at −80°C.

After thawing, cells (10.7 g) were resuspended in lysis buffer (160 ml). Proteinase inhibitor cocktail “Complete” pills (Roche) were added according to the manufacturer’s prescription, and PMSF added to 0.7 mM just prior to the cells being lysed by being passed twice through a French press (12000 psi). The lysate was clarified by centrifugation (35000 × g; 30 min) to yield the soluble Fraction I. Proteins that were precipitated from Fraction I by addition of solid ammonium sulfate (0.4 g/ml) and stirring for 60 min, were collected by centrifugation (35000 × g; 30 min) and dissolved in buffer A+150 mM NaCl (40 ml). The solution was dialyzed against three 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 with buffer A+150 mM NaCl. Fractions containing proteins that did not bind to the column were pooled and dialyzed against three changes of 2 l of buffer A.

The dialysate (Fraction III, 50 ml) was loaded at a flow rate of 2 ml/min onto a column (2.5 × 16 cm) of the same resin, now equilibrated in buffer A. Again, fractions containing proteins that did not bind to the column were pooled to yield Fraction IV (50 ml). Fraction IV was applied at 1 ml/min to a column (2.5 × 16 cm) of SP-Sepharose fast flow resin (Amersham Biosciences) that had been equilibrated in buffer A+50 mM NaCl. After the column had been washed with 80 ml of the same buffer, $\tau_{C22}$ was eluted using a linear gradient (500 ml) of 50–450 mM NaCl in buffer A. It eluted in a single peak at about 150 mM NaCl. Fractions containing purified $\tau_{C22}$ were pooled and dialyzed against three changes of 2 l of buffer GF, to give Fraction V (30 ml, containing 30 mg of protein). Aliquots were frozen in liquid nitrogen and stored at −80°C.

ESI-MS gave a value of 22089 ± 1 for the molecular weight of $\tau_{C22}$. This molecular weight corresponds to the value calculated for 98.6% $^{15}$N enrichment in the presence of an uncleaved methionine.
REFERENCES

S1. Studier, F.W. (2005) Protein production by auto-induction in high-density shaking cultures. Protein Express. Purif., 41, 207–234.

S2. Tsuchihashi, Z. and Brown, P.O. (1992) Sequence requirements for efficient translational frameshifting in the Escherichia coli dnaX gene and the role of an unstable interaction between tRNA^{Lys} and an AAG lysine codon. Genes Dev., 6, 511–519.

S3. Ozawa, K., Jergic, S., Crowther, J.A., Thompson, P.R., Wijffels, G., Otting, G. and Dixon, N.E. (2005) Cell-free protein synthesis in an autoinduction system for NMR studies of protein-protein interactions. J. Biomol. NMR, 32, 235–241.

S4. Neylon, C., Brown, S.E., Kralicek, A.V., Miles, C.S., Love, C.A. and Dixon, N.E. (2000) Interaction of the Escherichia coli replication terminator protein (Tus) with DNA: a model derived from DNA-binding studies of mutant proteins by surface plasmon resonance. Biochemistry, 39, 11989–11999.

S5. Love, C.A., Lilley, P.E. and Dixon, N.E. (1996) Stable high-copy-number bacteriophage λ promoter vectors for overproduction of proteins in Escherichia coli. Gene, 176, 49–53.

S6. Beckett, D., Kovaleva, E. and Schatz, P.J. (1999) A minimal peptide substrate in biotin holoenzyme synthetase-catalyzed biotinylation. Protein Sci., 8, 921–929.

S7. Vasudevan, S.G., Armarego, W.L.F., Shaw, D.C., Lilley, P.E., Dixon, N.E. and Poole, R.K. (1991) Isolation and nucleotide sequence of the hmp gene that encodes a haemoglobin-like protein in Escherichia coli K-12. Mol. Gen. Genet., 226, 49–58.

S8. McIver, L., Baxter, R.L. and Campopiano, D.J. (2000) Identification of the [Fe-S] cluster-binding residues of Escherichia coli biotin synthase. J. Biol. Chem., 275, 13888–13894.

S9. Crowther, J.A. (2000) Ph.D. Thesis, the Australian National University.
Figure S6. $\tau_{C16}$ can be purified from its proteolytic fragments by anion exchange chromatography. (A) A sample of $\tau_{C16}$ (6 ml; 3.2 mg/ml) that had undergone partial proteolysis was dialyzed against three changes of 2 l of buffer A, then applied at a flow rate of 1 ml/min onto a Mono Q HR 10/10 column (Pharmacia). The $\tau_{C16}$ protein was resolved from its proteolytic fragments using a linear gradient (45 ml) of 0–250 mM NaCl in buffer A. Fractions (0.5 ml) were collected. (B) Fractions, analyzed by 15 % SDS-PAGE, show that while proteolytic fragments elute earlier within peaks 1 and 2, $\tau_{C16}$ elutes later within peak 3. ESI-MS analysis indicated that protein in peak 1 corresponded to $\tau_{C14}$. Only the relevant portion of the gel is shown.
Figure S7. Deletion mutants τC16Δ11 and τC16Δ18 (τC14) do not form a stable complex with α. Mixtures of α with τC14 (A) and τC16Δ11 (B) proteins were resolved by gel filtration on a Sephacryl S-100 HR column as described in Figure 2. Proteins in successive fractions were analyzed by 15% SDS-PAGE. No stable interaction between α and either of the two deletion mutants was detected.
**Figure S8.** Competititv SPR measurements of the interactions between $\alpha$ and $\tau_{C16}$ (A), $\tau_{C\text{pep}32}$ (B) and $\tau_{C16\Delta7}$ (C). Sensorgrams showing interaction of 1 nM $\alpha$ in the presence of increasing concentrations (from top to bottom) of competitor proteins with immobilized bio-$\tau_{C16}$ are shown in coloured curves at the left. Curves were fit individually to a 1:1 Langmuir binding model with mass transfer using BIAevaluation 3.1 software as described in Materials and Methods, to yield values of $K_{D,\text{app}}$. Curves simulated on the basis of derived parameters are shown in black. At the right are shown plots of experimentally determined $K_{D,\text{app}}$ values versus concentrations of competitor proteins, as indicated (note the different units). (A) $\tau_{C16}$ was used at concentrations of 0, 100, 150, 300, 500 and 1000 pM; slope $m$ and intercept $c$ of the plot at the right were $0.397 \pm 0.037$ and $(0.53 \pm 0.10) \times 10^{-10}$ M, respectively. (B) $[\tau_{C\text{pep}32}]$ were 0, 5, 10, 20 µM; $m = (0.371 \pm 0.004) \times 10^{-4}$; $c = (1.43 \pm 0.04) \times 10^{-10}$ M. (C) $[\tau_{C16\Delta7}]$ were 0, 5, 10, 20 µM; $m = (0.130 \pm 0.013) \times 10^{-4}$; $c = (0.87 \pm 0.15) \times 10^{-10}$ M. The derived $K_D$ values for the interaction of $\alpha$ with each of the competitor proteins are given in Table 1. Measurements were carried out in SPR buffer at 20°C.
Figure S9. Interactions between $\alpha$ and $\tau_{C16}$ single point mutants were investigated by analytical gel filtration. Complexes of $\alpha$ and $\tau_{C16}[S617P]$ (A), $\tau_{C16}[I618T]$ (B), $\tau_{C16}[L627P]$ (C), $\tau_{C16}[F631I]$ (D), $\tau_{C16}[L635P]$ (E), and $\tau_{C16}[D636G]$ (F) were resolved from excess amounts of $\tau_{C16}$ single point mutant proteins on a Sephacryl S-100 HR analytical gel filtration column. In each case, samples prepared as described in Materials and Methods were analyzed on a 15% SDS-PAGE gel stained with Coomassie brilliant blue. Successive fractions from each chromatogram are shown from left to right.
Figure S10. BIACORE sensorgrams showing association and dissociation phases of α–bio-τc16 interactions in the presence of competitive inhibitors: τc16[S617P], τc16[I618T], τc16[L627P], τc16[F631I], τc16[L635P], and τc16[D636G], as indicated. Solutions of 1 nM α were mixed with concentrations shown of τc16 point mutant proteins and tested against the interaction with immobilized bio-τc16. Curves, shown in colours, were fitted individually to a Langmuir one-to-one with mass transfer model as described in Section 5.2.13. The variables $R_{max}$ (66.3 RU) and $k_d$ ($7.00 \times 10^{-3} \text{ s}^{-1}$) determined by fitting of the sensorogram when there was no inhibitor present in the analyte were set as constants in three other individual fits for each inhibitor (see Materials and Methods). Sensorograms were simulated (black curves) using the same model and the fitted parameters (see Figure S8). Measurements were carried out at 20°C.
Figure S11. $K_D$ values for interaction of $\alpha$ with mutant versions of $\tau_{C16}$. $K_{D,app}$ values of $\alpha$–bio–$\tau_{C16}$ interaction determined by BIACORE (Figure S7) were plotted against concentrations of competitive inhibitors ($\tau_{C16}$ mutant proteins): $\tau_{C16}[S617P]$, $\tau_{C16}[I618T]$, $\tau_{C16}[L627P]$, $\tau_{C16}[F631I]$, $\tau_{C16}[L635P]$, and $\tau_{C16}[D636G]$, as indicated. The least-squares lines give values of the slopes and intercepts used to calculate $K_D(\alpha-I)$ values (Table 1). Errors were calculated using the program ORIGIN (OriginLab Corporation, Northampton, USA). Scales on the x-axis differ due to different ranges of concentrations of $\tau_{C16}$ mutant proteins needed for inhibition of the $\alpha$–bio–$\tau_{C16}$ interaction.