Escherichia coli

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THE sequences called Ter sites, located in the terminus region of the chromosome, and forms replication-arrest complexes that block movement of DNA replication forks in a polar fashion. We have analyzed Tus to determine some of its physical parameters and biochemical characteristics. Native Tus had an 

molar absorption coefficient of 39,700 cm$^{-1}$ cm$^{-1}$. The data also indicated that Tus existed as a monomeric protein in solution and when complexed with its cognate DNA binding site. Secondary structure estimated from the circular dichroism spectrum suggested that Tus consisted of 40% α-helix, 0% β-sheet, 15% turn, and 45% aperiodic structure. The isoelectric point of native Tus (pH 7.5) was significantly different than that calculated from its amino acid sequence (pH 10.1), possibly because the tertiary structure of Tus perturbs the ionization of several residues. In addition, partial proteolytic digests of free Tus protein did not produce a subfragment of Tus that retained DNA binding activity, but did demonstrate that Tus was resistant to proteolysis when complexed with a Ter site.

In the circular chromosome of Escherichia coli, replication forks move unimpeded from the replication origin, oriC, toward the terminus region but are arrested when moving in the terminus-to-origin direction. Arrest of DNA replication is mediated by protein-DNA complexes comprised of specific chromosomal DNA sequences, called Ter sites, that are bound by the protein Tus (1–3). The orientation of a Tus-Ter site protein-DNA complex is critical for its function; in one orientation it allows the replication fork to pass freely, while in the other orientation the replication fork is arrested (4, 5). Six Ter sites have been identified in E. coli, three in each half of the chromosome. TerA, TerD, and TerE arrest replication forks moving from the terminus to the origin in the counterclockwise direction; TerB, TerC, and TerF arrest clockwise traveling replication forks (reviewed in Ref. 6). In addition, binding of Tus to the TerB site regulates transcription of the tus gene (7, 8).

The molecular mechanism by which DNA replication is halted by a Tus-Ter complex is currently unknown, but two models have been considered (9, 10). One model is that Tus mediates replication fork arrest through specific protein-protein interactions with a component of the replication apparatus, most likely the DnaB helicase. The second model is that Tus acts as a simple clamp on the DNA and forms a polar barrier to the progression of DNA replication. In vitro studies using purified Tus protein in strand displacement assays have suggested that Tus has both specific (protein-protein) and nonspecific (barrier) activities against proteins that translocate along DNA. Nonspecific Tus activities include orientation-independent arrest of several polymerases (11) and orientation-dependent arrest of a variety of helicases (9, 10), including eukaryotic helicases (12, 13). Evidence supporting specific activities of Tus includes the inability to arrest certain helicases (12, 14), inactivity against DnaB on elongated substrates (14), and recently, the discovery of an anti-terminator that abrogates the anti-helicase activity of Tus without displacing Tus from its binding site (15).

Based on the sequence of the tus gene, the Tus protein contains 309 amino acids and has a molecular mass of approximately 36 kDa (1, 16). Amino-terminal sequencing and acid hydrolysis of purified protein has confirmed the composition of Tus (16). Analysis of the amino acid sequence has suggested that (1) Tus does not appear to contain any of the common DNA-binding motifs, and (2) it has a significantly greater number of basic than acidic amino acid residues, with a predicted isoelectric point of approximately 10.1 (1). Studies of Tus affinity for the chromosomal Ter sites have shown that Tus binds to TerB or TerF with a $K_d$ value of $3 \times 10^{-13}$ M and $1 \times 10^{-11}$ M, respectively, and forms a stable complex with the Ter sites, having a half-life in vitro of 550 min for TerB (17) and 45 min for TerF (18). Finally, Tus has been reported to exist as a monomer in solution (19), but the stoichiometry of DNA binding is less certain, as it has been suggested that Tus binds to a Ter site as either a dimer (3) or a monomer (19).

In this report, we present an analysis of the Tus protein, which was undertaken to determine some of its physical parameters and biochemical characteristics. The sedimentation coefficient, Stokes' radius, frictional coefficient, axial ratio, isoelectric point, and molar absorption coefficient of Tus were determined. It was also demonstrated that Tus existed as a monomer both in solution and when bound to a Ter site. In addition, a circular dichroism spectrum of purified Tus suggested that approximately 40% of the protein existed in the form of an α-helix, 0% in β-sheet, 15% in turns, and 45% in aperiodic structures. Last, it was shown that Tus was resistant to proteolytic digest when complexed with a Ter site, but it was not possible to separate DNA binding and replication arrest domains of Tus by partial proteolysis.

MATERIALS AND METHODS

Purification of Tus Protein—Tus protein, Fraction 6A, was purified from strain TH241 as previously described (20), with an additional fractionation step using Sephacryl S-100. The activity of Tus was de-
termed using a filter binding assay (17).

**Ultraviolet Absorption of Tus**—After extensive dialysis against water adjusted to pH 7.0-7.5 with NaOH, approximately 5 mg of purified Tus was dialyzed at 75 °C in a separatory funnel. Dry weighed volumes of 3 ml of Tus protein was dissolved in 1 ml of 0.02 M NaPO₄ buffer (pH 7.4) containing 0.15 M NaCl. Since Tus precipitated out of solution over time at this concentration in this buffer, a few microliters of dithiothreitol were added to maintain protein solubility. The final pH of the solution was roughly 4. This solution was then used to prepare serial dilutions of Tus, and the optical density for each diluted Tus sample was measured at 205, 210, 215, 225, and 280 nm. Alternately, the concentration of Tus was determined by measuring its absorption at 205 nm, where absorption is due primarily to the peptide bonds rather than aromatic compounds (21). 1 mg/ml Tus was dissolved in 0.05 M NaC1, 0.05 M Tris buffer (pH 7.4) containing 1.0 mg/ml blue dextran 2000, buffered in 25% acetic acid, and then destained in 10% MeOH, 7.5% acetic acid.

**Sucrose Density Gradient Centrifugation**—3-ml linear gradients were prepared from 5-20% sucrose solutions in 0.05 M Tris-C1 (pH 7.5) containing 0.05 M NaCl, 1.0 mg/ml dithiothreitol, and 1.0 mg/ml EDTA. Gradients were stored for 4-10 h at 4 °C before use. A mixture containing 0.2 mg of a standard protein of known sedimentation coefficient, either bovine serum albumin or ovalbumin, and 0.01 mg of Tus protein in 0.2 ml of buffer was carefully layered onto the sucrose gradient. In studies using chymotrypsinogen as the standard, the chymotrypsinogen was loaded onto a separate gradient from Tus. Samples were centrifuged at 50,000 rpm in a Sorvall TST 60.4 swinging-bucket rotor for 20 h at 4 °C. Fractions of 0.1 ml were collected from the bottom of the tubes and fractions containing the standards were identified using the method of Bradford (22). Peak fractions of the standard were identified using a filter binding technique.

The molecular mass and sedimentation coefficients of the standard proteins used for these studies were 66,500 Da and 4.58 S for bovine serum albumin, 43,500 Da and 3.55 S for ovalbumin, and 24,325 Da and 2.58 S for chymotrypsinogen A. These values were obtained primarily from reference (23). However, in the literature, sedimentation coefficients are given for sera with values of 4.2 to 5.5 S. The above values are not corrected for the effect of the protein solution on the figure given above represents an average value. The concentration of the Tus/terB oligomer was first determined by measuring its absorption at 260 nm, and 0.55 μg of Tus was then labeled with 32P using γ-32P-ATP (5000 Ci/mmol; Amersham Corp.) and T4 DNA kinase (Life Technologies Inc.). Unincorporated nucleotides were removed by passing the reaction mix through a 1-ml Sephadex G-50 column, and the radiolabeled Tus (1-2 × 10⁶ cpm/μl or 820 Ci/μmol) was then diluted with Tris-EDTA buffer to 1 × 10⁴ cpm/μl. 10 μl of the diluted, labeled Tus were mixed with 10 μl of unlabelled Tus (0.55 μg/μl) and an aliquot was counted to obtain the specific radioactivity of the Tus/terB mix prior to use in the gel retardation assay.

**Gel Electrophoresis and Staining**—As previously described for Tus/terB complex, the method of Lewis et al. (24) was employed with the following modifications. Twenty-five pmol each of [3H]Tus and [32P]TerB were mixed together with an equal volume of 2 × binding buffer (100 mM Tris-C1 (pH 7.5), 300 mM potassium glutamate, 0.2 mM EDTA, 0.2 mM dithiothreitol, and 200 μg/ml BSA) and incubated at 37 °C for 45 min at room temperature. The final reaction volume was 15 μl. Ths (100 μg/ml Tris-EDTA buffer, 0.05% bromphenol blue, 0.025% xylene cyanol, 50% glycerol, and 0.125 μEDTA) were added to the samples before loading onto a 3% NuSieve agarose gel (FMC Bio Products). Electrophoresis was performed in a horizontal mini-gel apparatus at 100 V for 1 h; the gel was then stained with ethidium bromide before photography. The retarded band containing the Tus/terB complex was cut out of the gel, transferred to a microcentrifuge tube, incubated at 65 °C for 10 min, and then transferred to 42 °C for 5 min. 0.10 volume of 10 × β-agarase buffer was added, and the reaction was continued at 42 °C for 45 min. Tus/terB was then mixed with 15 μl of the scintillation mixture and counted. After subtracting background counts and correcting for spillover of 32P into the 3H channel, the ratio of 3H cpm/32P cpm was calculated.

**Isoelectric Focusing**—Acetylated cytochrome c protein standards having pl values of 4.1, 4.9, 5.4, 6.3, 9.7, and 10.6 were purchased from U. S. Biochemical Corp. Polyacrylamide gels containing 7.5% acrylamide (29:1), and 2% ampholine, pH 3.5-10 (Pharmacia) were cast in 0.5 inch-glass gel tubes. 40 μl of a solution containing a mixture of the protein standards (20 μg of each standard) were mixed with 60 μl of sample buffer (60% glycerol, 4% ampholine, pH 3.5-10) and layered over the gel surface. Purified Tus protein samples (2 μg of protein in 20 μl of water) were loaded with and without standards. A protective zone of 50 μl of 15% glycerol, 2% ampholine (pH 3.5-10), and 1% L-glutamic acid was then applied, and the gel tubes were filled to the top with 20 μl phosphoric acid. Electrophoresis was conducted at a constant voltage of 200 V for 5 h at 4 °C with 20 μl phosphoric acid in the anode reservoir and 1 × NaOH in the cathode reservoir. Gels were stained with 0.2% (w/v) Coomassie Blue G-250 in 50% MeOH, 7.5% acetic acid, and then destained in 10% MeOH, 7.5% acetic acid.

To assay Tus binding activity, an unstained isoelectric focusing gel was cut into 0.5-cm sections, which were then minced with a razor blade. The protein was eluted from the gel fragment in 150 μl of KG binding buffer (50 mM Tris-C1, pH 7.5, 150 mM potassium glutamate, 0.1 mM dithiothreitol, 0.1 mM EDTA, and 100 μg/ml BSA) at 4 °C for 40 h. Aliquots of the eluant were assayed using the filter binding technique.

**Gel Chromatography**—The CD spectra of Tus protein in 10 mM Tris-borate buffer, pH 8.3, containing 40 mM KCl, was recorded at 20 °C using a 0.01-cm path length cell on a Jasco J-710 circular dichroism spectrometer. The spectrometer was calibrated using an aqueous solution of (+)-10-camphorsulfonic acid (Aldrich) as described by Johnson (25). The spectrum shown is the average of three scans and is not smoothed.

**Proteolytic Digest of Tus**—30 μg of purified Tus were suspended in TMB buffer (50 mM Tris-C1, pH 7.5, 5 mM MgCl₂, 25% glycerol) at 4 °C, and 1.5 μg of chymotrypsin (or trypsin) were added to give a final volume of 64 μl. Aliquots (1 μl) were removed from the digestion mixture at the indicated time points, added to 8 μl of PAGe sample buffer (0.125 mM Tris-C1, pH 6.8, 10% 2-mercaptoethanol, 6% SDS, 0.05% bromphenol blue, and 20% glycerol), and boiled for 5 min to terminate the reaction. Samples were then subjected to electrophoresis on a 15% SDS-PAGE gel. Following electrophoresis and staining the gels were scanned with a laser densitometer and the amount of intact Tus remaining for each time point was expressed relative to the amount of Tus present in the 0-min lane. Proteolytic digests of prebound Tus were done in a similar manner, except that a 1.5 molar excess of Tus/terB oligomer was added to the reaction 1 h prior to addition of the protease.

To determine the binding activity of Tus after partial digestion with either trypsin or chymotrypsin, the reaction was set up as described above, except that the 8-μl aliquots were added to tubes containing 2 μl of trypsin/chymotrypsin inhibitor (10 μg/ml; Sigma) to terminate the reaction. A portion of each sample was removed to determine Tus bind-
**Table I**

| Method | $A_{260}^{1\text{cm}^2}$ | $\varepsilon_{260}$ Reference |
|--------|-------------------|-----------------------|
| 205    | 1.15              | 41.170                |
| 205/280| 1.07              | 38.163                |
| 210    | 1.01              | 36.158                |
| 215/225| 1.01              | 36.158                |

**RESULTS**

Determination of the Extinction Coefficient of Tus—The molar absorbance coefficient ($\varepsilon_{260}$) was determined by preparing a solution containing 1 mg/ml of Tus protein, making serial dilutions ranging from 10 to 100 $\mu$g/ml and measuring the absorbance of these diluted samples at wavelengths of 205, 210, 215, 225, and 280 nm. We then plotted the observed absorption values versus the protein concentration of the different Tus dilutions, and a least squares analysis of the data was used to calculate the expected absorbance of a 1 mg/ml solution of Tus protein at each wavelength. The correlation coefficient ($r$) of the derived lines in all cases exceeded 0.99. The $A_{260}^{1\text{cm}^2}$ values obtained were $31.8 \pm 1.5$ for $A_{260}$, $22.1 \pm 1.1$ for $A_{210}$, $16.4 \pm 0.1$ for $A_{215}$, $9.4 \pm 0.2$ for $A_{229}$, and $1.09 \pm 0.03$ for $A_{280}$. These values were then used to calculate the extinction coefficient using the 205-nm method of Van Iersel et al. (26), Scopes' 205/280-nm method (21), Tomb's 210-nm method (27), and Wadell's 215/225-nm method (28). The values for $A_{260}^{1\text{cm}^2}$ obtained were similar regardless of the method used, ranging from 1.01 to 1.15 (Table I).

Sucrose Gradient Centrifugation—The sedimentation coefficient ($s_{20, w}$) and approximate molecular mass of native Tus were determined by comparing its sedimentation properties to those of three standard proteins during sucrose gradient centrifugation. 5-20% sucrose gradients containing either a mix of Tus and the standard or with the standards and Tus loaded onto separate gradients were centrifuged as described under "Materials and Methods." Fig. 1 shows a typical sedimentation pattern for Tus and BSA and also a plot of the S value of the standards and Tus versus their positions in the gradient. Based the method of Martin and Ames (29), we used a least squares plot of the data in Fig. 1 to determine that the $s_{20, w}$ of Tus was $3.2 \text{ S}$. In addition, we estimated that native Tus had a molecular mass of 36,190 Da. This value was in good agreement with the predicted molecular mass of Tus based on its amino acid composition (35,783 Da) and suggested that Tus was a monomer in solution.

Gel Filtration of Tus—To determine the Stokes' radius, purified Tus was chromatographed on a Sephacryl S-100 column that had been standardized previously with a series of proteins of known Stokes' radii. The elution volume of Tus relative to the standard proteins was first used to calculate $K_w$, the elution volume parameter, and then a calibration curve was constructed by plotting $-\log K_w^{1/2}$ versus Stokes' radius (30) to determine the Stokes' radius of Tus.

The void volume of the column was $80 \text{ ml}$, and the elution volumes of the standard proteins measured from the initial addition of protein sample to the peak of the eluted protein were as follows: bovine serum albumin, $92 \text{ ml}$; ovalbumin, $99 \text{ ml}$; chymotrypsinogen A, $115 \text{ ml}$; and ribonuclease A, $128 \text{ ml}$. The elution volume of Tus was $111 \text{ ml}$. All peaks appeared to be sharp and symmetrical as shown in Fig. 2 and the calculated $K_w$ values for the standard proteins were $0.10$ for bovine serum albumin, $0.16$ for ovalbumin, $0.30$ for chymotrypsinogen A, and $0.41$ for ribonuclease A. When plotted against the Stokes' radius, the standard proteins formed a consistent series (Fig. 3), with the calculated $K_w$ value of Tus (0.27) corresponding to an apparent Stokes' radius of $23.2 \pm 1.5 \text{ Å}$.

The values for frictional and axial ratios were also determined from gel filtration chromatography and permitted us to estimate the molecular shape of Tus. The frictional ratio ($f/f_0$) of Tus was calculated according to Seigel and Monty (31) and was 1.05, which corresponds to an axial ratio of 2 (32).

Stoichiometry of Tus-TerB Complex—A direct estimation of the number of Tus molecules bound to each TerB site was obtained using a gel retardation assay (24). For this purpose, Tus protein and the TerB oligomer (33 base pairs) were labeled with $^3\text{H}$ and $^32\text{P}$, respectively. Equal amounts (25 pmol) of $^3\text{H}$Tus and the TerB mix were combined, incubated for 45 min at room temperature, and subjected to electrophoresis. The gel was then stained with etidium bromide to visualize the DNA. Only a single retarded band containing the Tus-TerB complex was observed (data not shown), which was cut out of the gel, digested with $\beta$-agarase to eliminate the quenching effect of the agarose, mixed with scintillation cocktail, and counted for both isotopes. The results showed that Tus bound to TerB DNA in a 1.1 molar ratio (Table II), suggesting that Tus complexed with its binding site as a monomer.
Characteristics of Tus

**Determination of the Isoelectric Point of Tus**—The isoelectric point of Tus has only been estimated from its amino acid sequence and was reported to be 10.1 (1); consequently, we were interested in determining the actual isoelectric point of native Tus. Purified Tus, either alone or combined with the acetylated cytochrome c standards, was layered onto polyacrylamide gels with a pH of approximately 7.5, indicating that this was in fact the isoelectric point of native Tus.

**Secondary Structure of Tus**—The CD spectrum of Tus was characteristic of proteins containing α-helical secondary structure (Fig. 6). To estimate the percentages of the various types of secondary structure, the CD spectrum of Tus was fitted to a linear combination of basis spectra for helix, β-sheet, turn, and random coil (33) using an algorithm that fits the data to the extrema and crossover points in the spectrum. The fitted spectrum calculated from the basis spectra (Fig. 6) consisted of 40% α-helix, 0% β-sheet, 15% turn, and 45% aperiodic structure and agreed well with the observed spectrum.

**Partial Proteolytic Digests of Tus**—Since the Tus protein has two known activities, DNA binding and arrest of DNA replication, we attempted to determine if these functions were located on separate domains by performing a partial proteolytic digestion. Tus was incubated with either trypsin or chymotrypsin for varying lengths of time, and aliquots were prepared for either SDS-PAGE electrophoresis, to determine the amount of intact Tus remaining, or for a filter-binding assay, to determine the amount of Tus DNA binding activity remaining.

Digestion with chymotrypsin produced one rather stable subfragment of 32 kDa that was the predominant species from 2 to 20 min and a second subfragment of 25 kDa that was the predominant subspecies at 40 and 60 min (Fig. 7). However, neither of these subfragments were able to bind to the TerB fragment as the loss of binding activity correlated with loss of intact Tus (Fig. 8). When digested with trypsin, Tus was rapidly converted to several smaller subspecies ranging in molecular mass from 20–32 kDa (Fig. 7). The loss of binding activity during tryptic digest again correlated with loss of intact Tus, indicating that none of the subfragments retained binding activity (data not shown).

We also performed the proteolytic digests when Tus had been prebound to a 33-base pair oligomer containing the TerB site to determine if the pattern of subfragments changed following Tus binding. As shown in Fig. 7, Tus was well protected from the action of both trypsin and chymotrypsin, with only a fraction of the total protein digested by the proteases. We also

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3 G. R. Moe, unpublished results.
observed that the fraction of Tus that was digested had the same pattern of subfragments as unbound Tus. We speculate that the fraction of Tus digested in these experiments represents protein that had dissociated during the incubation, suggesting that the primary sites of digestion by the two proteases were obscured by the bound DNA.

**DISCUSSION**

The Tus protein of *E. coli* is a DNA-binding protein capable of arresting DNA replication in a polar fashion when bound to any of the several Ter sites located around the chromosome. Tus binds to its recognition sequences with a high affinity and is the only known DNA-binding protein capable of halting DNA replication in *E. coli*. The unusual activity of this protein has led to several investigations of its ability to arrest the action of helicases and polymerases, but little is known of the physical and biochemical characteristics of Tus other than those that could be gleaned from the predicted amino acid sequence derived from the gene. Therefore, as part of an ongoing effort to characterize the Tus protein, we have determined its extinction coefficient, size, shape, helical content, and isoelectric point. In addition, we have performed proteolytic digests in an effort to separate functional domains in the polypeptide.

To determine the extinction coefficient of Tus, direct spectrophotometric measurements of Tus absorbance at different wavelengths were collected and several methods were then used to estimate the $\varepsilon_{280}$. All of these methods gave similar results, producing $A_{280}$ values that ranged from 1.0 to 1.15 and $\varepsilon_{280}$ values of 36,500–41,200 M$^{-1}$ cm$^{-1}$. However, based on the analysis by Van Iersel et al. (26), who demonstrated that the 205-nm method and the 205/280-nm method were superior for determining extinction coefficients, we averaged the numbers obtained by these two methods to arrive at our final values, which were an $A_{280}$ of 1.11 and $\varepsilon_{280}$ of 39,700 M$^{-1}$ cm$^{-1}$.

We also calculated the $\varepsilon_{280}$ of Tus according to the method of Wetlaufer (34), where the sum of the absorption of the individual amino acids of a protein was considered roughly equivalent to the protein's total UV absorption spectrum. For Tus, the molar absorption values for the amino acids tyrosine ($\varepsilon_{276} = 1340$), tryptophan ($\varepsilon_{278} = 5550$), and phenylalanine ($\varepsilon_{271} = 10$) were multiplied by the number of these residues in the protein, which were 8, 5, and 8, respectively. The calculated value for the $\varepsilon_{280}$ of Tus was 38,550 M$^{-1}$ cm$^{-1}$, which was very similar to the value obtained from our spectrophotometric measurements.

The sedimentation coefficient of native Tus was determined by centrifugation in sucrose gradients with standards of known $S$ values. Tus showed a single major peak corresponding to an $s_{20,w}$ of 3.2 S and an apparent molecular mass of 36,190 Da, suggesting that the protein remains as a monomer in solution,
**FIG. 7. Partial proteolysis of Tus.**

*Top,* digestion of Tus with chymotrypsin or trypsin. 30 pg of Tus were digested with 1.5 pg of either trypsin or chymotrypsin. Samples were prepared for electrophoresis at the indicated times (number of minutes) after addition of protease.

*Bottom,* protection of Tus when bound by DNA. 30 pg of Tus were preincubated for 1 h with a 1.5 molar excess of DNA containing the TerB site. 1.5 pg of chymotrypsin or trypsin was added and aliquots were removed and prepared for electrophoresis at the indicated times (number of minutes).

Characteristics of Tus

in agreement with previous column chromatography studies by Sista *et al.* (19). It should also be noted that, in addition to gradients that contained a mix of proteins, sedimentation experiments were also performed with the proteins in individual gradients to eliminate any possible protein-protein interactions that could influence the results. We observed that Tus exhibited the same sedimentation behavior when mixed or when centrifuged in separate gradients.

Gel filtration experiments provided a means to determine the Stokes’ radius of Tus, which was estimated to be 23.2 ± 1.5 Å. This result suggested that a single monomer of Tus would cover approximately 13 base pairs when bound to B-form DNA. This finding is consistent with the results from protection and footprinting experiments (17) that showed that the DNA-protein contacts between the Tus and TerB site were concentrated within the 11-base pair core sequence. In addition, the frictional ratio (*f*/*f*₀) and shape of Tus were determined from the Stokes’ radius using the equations of Siegel and Monty (31). Tus had a *f*/*f*₀ value of 1.05, corresponding to an axial ratio of 2 and indicating that the long axis of Tus is twice that of its short axis. This result suggested that Tus was ovoid shaped, but cannot tell us whether the shape of Tus was symmetric or asymmetric.

Reports regarding the stoichiometry of Tus binding to the Ter sites have suggested that Tus binds to DNA as either a dimer (3) or a monomer (19). This discrepancy may simply reflect the techniques used to make the stoichiometric determination. The relative electrophoretic position of free and bound DNA (35), which was one of the methods employed for these studies, is influenced by many factors, including the conditions of electrophoresis, the length of the DNA fragment, conformational effects, and the charge on the DNA-binding protein (36). Thus, the results obtained by this method can often be misleading. For instance, depending on the polyacrylamide concentration and length of the DNA fragment containing the Ter site, we obtained results with this technique indicating a monomeric, dimeric, or even trimeric Tus bound to the Ter site (data not shown). A second method, protein-protein cross-linking, was also used to show that multiple copies of Tus were not cross-linked (19), suggesting that Tus binds as a monomer to the R6KTer sites. However, the heterobifunctional cross-linking agent employed in these studies, sulfomaleimidobenzoyl-N-hydroxysuccinimide ester, may have been unable to cross-link adjacent Tus molecules. Sulfomaleimidobenzoyl-N-hydroxysuccinimide ester is 9.9 Å in length and has a reactive group for primary amines and a reactive group for free sulfhydryls; thus, cross-linking generally occurs between a lysine residue on one protein and a cysteine residue on the second that are separated by no more than 10 Å. Since there are only 2 cysteine residues in the Tus protein and the spatial configuration of these residues is unknown, it is possible that the inability to detect cross-linking between Tus molecules was due to the inaccessibility of the 2 cysteine residues to the cross-linking agent, particularly when the protein was bound to a DNA molecule.

Consequently, we measured directly the binding of Tus to TerB using differentially radiolabeled protein and DNA, a tech-
have to have an altered pK, to produce the observed isoelectric shift in the PI of Tus: a post-translational modification such as phosphorylation of Tus would be necessary to cover the 11-base pair core sequence of a tightly bound nucleic acid, at least two other possibilities could account for the acidic shift in the PI. Thus, it is likely that the tertiary structure of Tus, the results indicated 36% α-helix, 17% turns, and 48% aperiodic structure. This is in reasonably good agreement with the results of the CD studies. The prediction from the amino acid sequence suggested that the helical structure was primarily in the amino-terminal half of the protein while the aperiodic structure was located in the carboxyl-terminal segment. The latter is evident in the relatively large number of proline (9 of 17 total) and glycine (4 of 8 total) residues located in the C-terminal third of the Tus amino acid sequence. These residues are most often observed in aperiodic structures.

We were unable to detect a proteolytic fragment of Tus that retained DNA binding activity, as loss of DNA binding activity correlated with loss of intact Tus during proteolytic digestion. Thus, the domain of the protein that interacts with the replication fork was not separable from the DNA binding domain by this technique. In addition, when Tus was bound by DNA, the primary sites of cleavage by trypsin and chymotrypsin became inaccessible, rendering Tus resistant to proteolytic digestion. The observed resistance to proteolysis may simply reflect blockage of the cleavage sites by the DNA or, alternatively, could be the result of a conformational change in Tus upon DNA binding. The first possibility is most likely the case, since the CD spectrum of Tus complexed with a TerB oligomer was nearly identical to the sum of the spectra of each component alone. This suggested that Tus did not undergo a substantial conformational alteration upon binding to DNA.

Although the general organization of the terminus regions of E. coli and Bacillus subtilis are similar, some striking differences in the replication-arrest systems of these two divergent eubacteria are now evident. It has been shown that arrest of replication is mediated in B. subtilis by the binding of the replication terminator protein (RTP) to two imperfect inverted repeats of 47 to 48 bp (IR I and IR II), located in the terminus region of the chromosome (42). As in E. coli, the RTP-IR complexes show polarity of function; however, RTP is smaller, with a molecular mass of 14,500 daltons (4) and has no significant amino acid homology with Tus (1). Sedimentation equilibrium experiments demonstrated that RTP exists as a dimer in its native configuration (4), in contrast to our studies that showed that Tus was a monomer. Likewise, gel retardation analysis of the interaction between RTP and the IR region containing both IR I and IR II established that each inverted repeat contained two distinct RTP binding sites and that the fully saturated complex contained seven to eight RTP monomers (24). This result suggested that one dimer binds to each RTP binding site, whereas these studies indicated that a monomer of Tus binds to each Ter site. In addition, both sites of the IR must be occupied by RTP for replication arrest to occur (43), in contrast to studies in E. coli that have shown that only a single Ter site is necessary for Tus function (3, 44, 45). The observations that (1) both RTP binding sites must be occupied and (2) that a loop is not formed in the DNA (24) suggests that replication arrest in B. subtilis must occur as a result of a sequential interaction of the

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![Graph](image)

**Fig. 8. Loss of Tus DNA-binding activity following partial proteolysis by chymotrypsin.** Tus was digested with chymotrypsin as described for Fig. 5, except that chymotrypsin inhibitor was used to terminate the digestion at the indicated time points. An aliquot from each time point was removed for determining the DNA-binding activity and the remainder of the sample was prepared for electrophoresis. Densitometer tracings of the bands containing intact Tus were used to quantitate the amount of Tus remaining at a given time point relative to the initial amount of Tus. Tus DNA-binding activity, as determined by a filter binding assay, was also expressed relative to the initial amount of binding activity.
replication fork with the RTP dimers; whereas in *E. coli*, a lone Tus monomer is sufficient. As more is learned of the two replication arrest systems, it will be possible to ascertain if the observed structural dissimilarities also lead to functional differences in the mechanism of replication arrest in Gram-positive and Gram-negative bacteria.

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