Identification of a Tus Protein Segment That Photo-Cross-Links with *TerB* DNA and Elucidation of the Role of Certain Thymine Methyl Groups in the Tus−*TerB* Complex Using Halogenated Uracil Analogues

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ABSTRACT: Six potential hydrophobic sites of the Tus−*TerB* complex were analyzed using bromodeoxyuridine and iododeoxyuridine as isosteric analogues of thymine. Analogues were incorporated at individual sites, and dissociation rates were measured in 150 mM potassium glutamate, pH 8.0, using a nitrocellulose filter assay. These halogenated analogues serve as a probe of the environment in which they reside. Our measurement revealed at least two types of responses. Three sites showed increases in stability with the introduction of the bromo and iodo derivatives. The enhanced stability is proposed to result from polar or charged molecules in the vicinity of the halogenated analogues through dipole−dipole, dipole−ion, or dipole-induced dipole interactions. The other three sites exhibited the opposite trend, being destabilized by the introduction of these analogues. The destabilizing effects are attributed to a hydrophobic environment which cannot accommodate polar molecules. The photoreactivity of these analogues was utilized to specifically cross-link the Tus protein and *TerB* DNA. Using the substitution of bromodeoxyuridine at position 8 in the *TerB* DNA, Tus protein was covalently attached to the DNA, and by trypsin digestion a fragment of Tus was isolated. Sequencing of the peptide fragment revealed a segment that matched the amino acid composition from 122−139 of the Tus protein.

Termination of replication in *Escherichia coli* is mediated by the specific binding of a protein called Tus to a series of DNA binding sites named *Ter* [for reviews, see Hill (1996) and Baker (1995)]. The complex impedes the progress of the DNA replication forks by a putative contrahelicase activity associated with Tus protein that inhibits DnaB function. Tus−*Ter*-dependent termination of replication has been demonstrated in an *in vitro* system as well. The six termination sites that have been identified operate in an orientation-dependent manner. How Tus protein impedes the progress of replication forks is currently unknown. Either the complex provides a stable energetic barrier to the helicase required for replication or protein−protein interactions between Tus and a target protein signal the end of replication (Gottlieb *et al.*, 1992; Hiasa & Marians, 1992; Hidaka *et al.*, 1992; Khatri *et al.*, 1989; Lee *et al.*, 1989; Skokotas *et al.*, 1994, 1995).

Tus binding to one of the termination sites, *TerB*, has been measured and exhibits a half-life of 240 min in 150 mM potassium glutamate, pH 8.0 (Duggan *et al.*, 1995). No information is currently available on the possible structural motif used by the protein to bind to the DNA (Hill *et al.*, 1989; Keumpel *et al.*, 1989) although some similarities to other DNA binding proteins have been noted (Nedved *et al.*, 1994). Attempts at generating a fragment of Tus which retains binding activity have been unsuccessful (Coskun-Ari *et al.*, 1994).

Selective single-site substitutions of modified nucleotides in the *TerB* binding site have defined a series of hydrophilic and hydrophobic groups on the DNA necessary for complex stability (Duggan *et al.*, 1995). The Tus−*TerB* complex appears to require both major and minor groove determinants to achieve the lowest free energy of binding. This includes six methyl groups of thymine which presumably maximize stability through hydrophobic interactions.

In the work presented below, we have further characterized the role of the methyl groups in stabilizing the interaction. The use of two different halogenated compounds, which have a substituent at position 5 of different size, electronegativity, and polarizablity, provided insight into the nature of the environment at these sites. Additionally, these analogues are photoactivatible and therefore were used to cross-link the Tus−*TerB* complex. In the absence of structural data, cross-linking provides a glimpse at the protein in close proximity to the binding site. We report here the sequence of the Tus protein that cross-links with the bromodeoxyuridine-substituted DNA at position 8 of *TerB*.

EXPERIMENTAL PROCEDURES

Tus Protein Preparation, Purification, and Activity. A cellular extract containing Tus protein was prepared and passed through a Bio-Rex (Bio-Rad) column as previously described by Hill and Marians (1990). Fractions demonstrating the ability to bind *TerB* DNA (peak fractions) were pooled and precipitated with 70% $(NH_4)_2SO_4$ and centrifuged, and the pellet was resuspended in 50 mM imidazole chloride, pH 6.8, 1 mM DTT, 1 mM EDTA, and 20% glycerol

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(imidazole buffer). Further purification was achieved by FPLC. The material was applied to a SP-Sepharose fast flow column (60 mL) and eluted with a gradient of 0−1000 mM NaCl in the above imidazole buffer. The peak fractions were pooled, dialyzed against imidazole buffer (no salt), and loaded onto a Mono-S column. A gradient of 180−330 mM NaCl was applied to the column, and the desired fractions were collected and pooled. SDS−gel electrophoresis of the material showed the Tus protein to be greater than 99% pure. The activity of the protein was confirmed by previously described methods and was greater than 80% of the calculated protein concentration (Duggan *et al.*, 1995). All protein concentrations used in the dissociation rate assay refer to the active protein concentration.

Synthesis and Purification of Oligonucleotides. All oligonucleotides were prepared by automated synthesis using an Applied Biosystems Inc. DNA synthesizer. The bromodeoxyuridine (BrU) and iododeoxyuridine (IU) phosphoramidites were purchased from Glen Research. Deprotection was achieved by incubation with 1 mL of ammonium hydroxide for 24 h at room temperature. The material was dried, dissolved in 80% formamide, and purified on a 20% polyacrylamide−7 M urea denaturing gel. The bands were detected by UV shadowing, but exposure was limited to 5 s for these light-sensitive analogues. Gel slices containing the DNA were extracted with Tris−EDTA (TE) buffer. The solution was desalted with a Sep Pak C18 column (Waters Chromatography). The concentration of each oligonucleotide was determined by its UV absorbance at 260 nm and by the calculated extinction coefficient for the DNA sequence.

DNA Duplex Preparation. DNA duplex formation was achieved according to procedures previously described (Duggan *et al.*, 1995). DNA used for dissociation studies was labeled by a fill-in reaction with the enzyme Sequenase (USB), [α-^{32}P]dATP, dCTP, dGTP, and TTP. Separation of the DNA from unreacted reagents was accomplished by filtration through a G-10 column (Gottlieb *et al.*, 1992).

For cross-linking experiments, single-stranded DNA containing the halogenated analogue was first labeled with T4 polynucleotide kinase (Promega) and [γ-^{32}P]ATP. The DNA was precipitated by the addition of sodium acetate (300 mM final concentration) and 2.5 volumes of ethanol, cooled to −20 °C overnight, and centrifuged at 14 000 rpm for 15 min. The pellet was washed with 70% ethanol and dried. The duplex DNA was formed by the addition of the complementary wild-type DNA in a solution of 150 mM NaCl at a final concentrations of 5000 pmol/50 μL. This solution was heated to 90 °C and slow-cooled.

Rate Dissociation Measurements. These kinetic constants for rate dissociation were determined by methods previously described (Duggan *et al.*, 1995). The data were fit to eq 1:

$$\ln \frac{\text{cpm} - \text{bkg}}{\text{cpm}_0 - \text{bkg}} = k_d t \qquad (1)$$

where cpm is the number of counts at time t, cpm_0 is the number of counts at time 0, k_d is the dissociation rate constant, and bkg is the number of background counts.

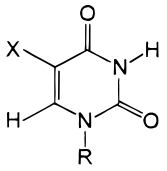
Photochemical Cross-Linking. Initial experiments were performed to optimize cross-linking parameters. Tus protein (1.0 μM) and derivatized duplex DNA (2.5 μM), containing a single substitution of BrU or IU for T, were incubated for 30 min in a solution containing 50 mM Tris-HCl, pH 8.0, 150 mM potassium glutamate, and 0.1 mM EDTA. The specific activity of labeled material ranged between 8.9×10^6 and 2.1×10^7 cpm per 1 pmol of DNA for these experiments. Twenty microliter aliquots were irradiated at a 312 nm wavelength (Hicke *et al.*, 1994). Samples were placed at varying distances from the source and treated with a range of dosages to identify optimal conditions for preparative irradiation. Subsequently, the samples were heated in Laemmli sample buffer at 90 °C for 2 min and electrophoresed on a 10% polyacrylamide−SDS gel (Laemmli, 1970).

Preparative Irradiation. Tus protein (25 nmol) was incubated with modified *Ter* DNA (62 nmol) in 1 mL of the above buffer. Increasing the concentrations from initial cross-linking experiments did not alter the efficiency of cross-link formation. Thirty microliter aliquots were irradiated for 30 min at a distance of 10 cm. The aliquots were then combined, placed in Laemmli sample buffer, and separated by SDS−PAGE. The cross-linked complexes were cut from a 10% polyacrylamide gel using an autoradiogram as a template. The gel slices were soaked in HPLC grade water, with three changes of water, over several days at 37 °C. The supernatants were combined and concentrated in a speed vac. More than 90% of the complex was recovered. The DNA complex was precipitated by the addition of 3 volumes of ethanol and 0.3 M NaOAc and placed at −20 °C for 18 h. After centrifugation and washing with 500 μL of 70% ethanol, the complex was resuspended in 100 mM Tris, pH 8.5.

Trypsin Digestion. After the removal of an aliqout, 20 μg of freshly resuspended modified sequencing grade trypsin (Boehringer Mannheim) was added to each vial and incubated at 37 °C. A second addition of enzyme was made after 24 h. The digestion was monitored with SDS−PAGE and was continued until a single fragment predominated. The sample volume was reduced to 200 μL.

Isolation of the Complex. The cross-linked complex was ethanol-precipitated with 2.5 volumes of ethanol and 0.3 M NaOAc at −20 °C for 18 h and separated on a 12% polyacrylamide−8 M urea gel with TBE used as the running buffer. Cyanate ions, which accumulate from the urea, could modify the N-terminal without the presence of a free radical scavenger. As a precaution, sodium thioglycolate was added to the upper buffer to a concentration of 0.1 mM. Amino-terminal blockage is also expected from nonpolymerized acrylamide monomer, reactive polymerization byproducts, peroxides, and free radicals. The gels were cast in advance up to 24 h ahead to decrease the amount free polyacrylamide and preelectrophoresed for a minimum of 2 h.

The samples were transferred electrophoretically by blotting onto a poly(vinylidene difluoride) membrane (Micron Separations Inc.) with a constant voltage of 20 V for 15 h in a Bio-Rad Trans Blotter (Hicke *et al.*, 1994). The transfer buffer used was 1× TBE with 0.1 mM thioglycolate and 10% methanol. The buffer was cooled to 4 °C, and the temperature in the transfer tank was maintained at 4 °C by circulating water through a cooling coil. The membrane portion containing the fragment was located through autoradiography and excised. The immobilized peptide was sequenced by automated Edman degradation.



	X	
Thymine	CH_3	2.0Å
Uracil	H	
Bromouracil	Br	1.95Å
Iodouracil	I	2.15Å

FIGURE 1: Chemical structures of bromodeoxyuridine and iododeoxyuridine used in this work. These are isosteric analogues for the thymine base also shown above. Uracil analogue is included for comparison purposes. van der Waals radii are indicated for each chemical group.

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              1   5    10   15   20
              |   |    |    |    |
5'AATTCATAAAATAAGTATGTTGTAACTAAAGTG
3'    GTATTTTATTCATACAACATTGATTTCACCTAG
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FIGURE 2: Sequence of the duplex DNA used for photo-cross-linking and rate dissociation measurements. Positions are identified by the numbers on top of the sequence. The nucleotide designated as 1 is the last nucleotide sequence in leading strand synthesis. The conserved region of the *Ter* binding site is indicated by the bar through the sequence.

RESULTS

Dissociation Rate Constants for IodoU- and BrU-Substituted DNA. The analogue BrU (Figure 1) was substituted for thymine in three positions in the top strand (9, 12, and 14) and three positions in the bottom strand (8, 16, and 19) (Figure 2 shows the sequence and numbering for the DNA referred to in this work). The dissociation rate of Tus from each modified *Ter* site was measured. We have shown in previous work that the dissociation rate is sufficient to characterize the stability of the complex (Duggan *et al.*, 1995). Substitutions at positions 14, 8, and 19 enhanced the stability beyond that of wild-type (Table 1) with half-lives of 422, 353, and 1070 min, respectively, as compared to the wild-type half-life of 240 min. The substitution at position 12 reconstituted wild-type binding. BrU replacement at positions 9 and 16 was less stable than wild-type with half-lives of 148 and 186 min (Table 1, Figure 3A), respectively. The dissociation rates always indicated a more stable interaction in the presence of the bromo group as compared to the complex with a uracil substitution at the same position (Duggan *et al.*, 1995). Presumably, by introducing a bromine atom, van der Waals contacts made between the protein and the DNA at position 5 of the pyrimidine ring were restored, and the necessary entropic rearrangements were minimized.

When the same six positions were replaced with IU (Figure 1), wild-type levels of binding were recovered for all positions but 12 and 16 (Table 1, Figure 3B). Not only did substitution at positions 14, 8, and 19 enhance stability beyond that of wild-type, but also beyond that of the BrU analogues at these sites. The half-lives observed for the analogues with the IU derivative at positions 9, 12, and 16 were shorter than those observed for the bromo derivatives, indicating greater instability (Table 1, Figure 3B). The most extreme example was the position 16 substitution which had a half-life of 44 min.

Formation and Characterization of UV Cross-Links. Cross-linking of protein and DNA was achieved by equilibrating the protein and DNA, and then irradiating at 312 nm. A dose-dependent specific complex was seen at two positions, 8 and 19, for both halogenated derivatives (Figure 4). The BrU analogs at these positions displayed cross-linking efficiency of at least 7−15% routinely, while cross-linking in the absence of the halogenated uracils was less than 0.5% at equivalent UV dosages. The presence of a BrU-independent cross-link proved that the protein−DNA contacts required for cross-linking were present in the wild-type complex and no major structural changes were occurring. The BrU serves to enhance the cross-linking. The efficiency was determined by cutting, counting, and quantitating the amount DNA, free and complexed. The percent of cross-linking, although significant, was not as great as seen previously when a laser had been used as the light source (Gott *et al.*, 1991; Hicke *et al.*, 1994). This lower extent of cross-linking is seen as the result of faster inactivation of the protein by the broader spectrum of the light from a traditional UV lamp. Irradiation of the protein alone before incubation with the DNA resulted in a decrease in cross-linking (data not shown). The efficiency of cross-linking is therefore dependent on the protein activity.

The protein−DNA complex was subjected to a range of dosages by varying exposure times, from 2 to 40 min, and by varying the distance of the source, from 5 to 25 cm. A 30 min exposure at 10 cm was determined to be the best combination, and the experiments described below were performed under these conditions. Irradiating DNA alone, in the absence of protein, showed no evidence of cross-linking.

The specificity of cross-linking was further established by measuring the change in the amount of cross-linking when an unlabeled *TerB* competitor was added to the sample and allowed to equilibrate before irradiation. The quantity of cross-linking is inversely proportional to the amount of unlabeled competitor, indicating that site-specific cross-linking is occurring (Figure 5, lane 2). When a nonspecific competitor was used similarly, no change in cross-linking was observed (Figure 5, lane 3).

The iodouracil-substituted analogues also showed enhanced sequence-specific cross-linking at the same positions. However, these complexes were sensitive to photodegradation and heating, and were not pursued in this work. Since BrU-containing DNA has been shown to be highly susceptible to UV-induced strand breakage (Hutchinson *et al.*, 1973; Ogata & Gilbert, 1977), by abstraction of a proton from a neighboring sugar, a similar degradation of the IU-containing DNA is anticipated. Willis *et al.* found that IU exhibits a 7-fold higher absorption probability than BrU. This would explain the greater degradation at equal UV doses.

We have concentrated our efforts on cross-linking effects between the Tus and *TerB* containing bromodeoxyuridine at position 8. When the complex was cross-linked at position 8, multiple bands were observed. The majority of the material migrated as a doublet although a third band was evident. Cross-linking has been shown to alter the mobility of proteins and nucleic acids (Steele & Nielson, 1978). To determine whether these bands were identical to each other but migrated differently because of multiple conformations, we isolated the doublet and the third band separately. Subsequent electrophoresis of these isolated materials revealed that their migration pattern was unaltered from the original gel. The isolated cross-linked complex showed constant breakdown into free DNA over time.

Initially we confirmed the presence of protein by subjecting the material to extensive proteolysis using proteinase K, and showed that the DNA liberated migrated identically to un-cross-linked DNA (data not shown). In order to identify

Table 1: Half-Life, Dissociation Rate, and Change in Free Energy for Thymine Substitutions with BrU and IU

	T → BrU			T → IU		
position	half-life (min)	dissociation rate[a] ($s^{-1} \times 10^{-5}$)	free energy change[b] (kcal/mol)	half-life (min)	dissociation rate ($s^{-1} \times 10^{-5}$)	free energy change (kcal/mol)
wild-type	240 ± 6	4.8 ± 0.1	−	240 ± 6	4.8 ± 0.1	−
8	353 ± 13	3.3 ± 0.1	−0.22 ± 0.005	394 ± 13	2.9 ± 0.1	−0.29 ± 0.008
9	148 ± 5	7.8 ± 0.3	0.29 ± 0.01	224 ± 10	5.2 ± 0.2	0.04 ± 0.01
12	241 ± 10	4.8 ± 0.2	−	102 ± 13	11 ± 0.2	0.49 ± 0.087
14	422 ± 9	2.7 ± 0.1	−0.34 ± 0.009	2315 ± 143	0.5 ± 0.03	−1.3 ± 0.05
16	186 ± 5	6.2 ± 0.2	0.15 ± 0.007	44 ± 0.2	26 ± 0.4	1.0 ± 0.003
19	1070 ± 107	1.1 ± 0.1	−0.87 ± 0.039	2222 ± 184	0.5 ± 0.01	−1.3 ± 0.04

[a] Dissociation rates were measured in 50 mM Tris, 150 mM KGlu, pH 8.0 and at 25 °C. The values derived represent at least three independent measurements and are tabulated above. [b] The change in the free energy of dissociation is based on the following equation: $\Delta\Delta G^{\ddagger} = -RT \ln (k_d^{wt}/k_d^{A})$, where $R = 1.987$ cal/(mol K) and $T = 298$ K.

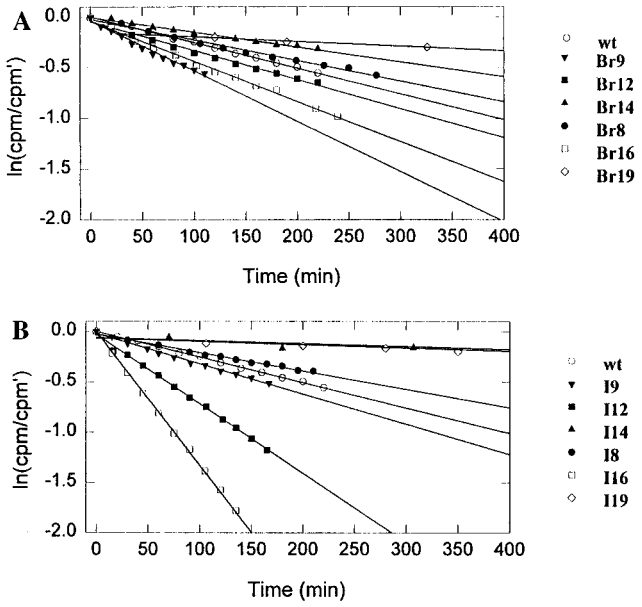

FIGURE 3: Graphical comparison of the dissociation rates for (A) BrU analogues and (B) IU analogues. Measurements were made in 50 mM Tris-HCl, pH 8.0 at 25 °C, 150 mM KGlu, 0.1 mM DTT, and 100 μg/mL BSA. The concentrations of the Tus protein and the *TerB* DNA were 1.5×10^{-11} M and 2×10^{-12} M, respectively. The data were plotted as first-order rate dissociations according to eq 1.

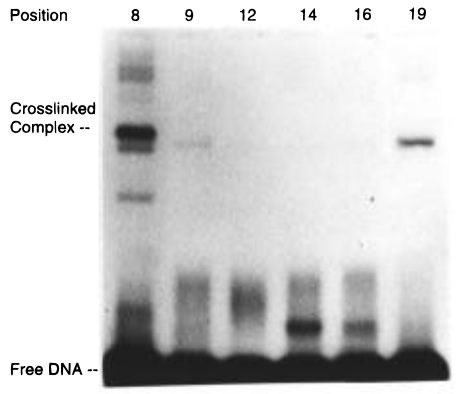

FIGURE 4: Site-specific photo-cross-linking of BrU singly substituted *Ter* DNA with Tus protein. Substituted ^{32}P-labeled DNA and Tus protein at 2.5 μM and 1.0 μM concentrations, respectively, were mixed and irradiated for 30 min at a distance of 10 cm with a 312 nm UV lamp. The irradiated samples were analyzed on a 10% SDS−polyacrylamide gel. The numbers above the gel indicate the position of the BrU substitution. Cross-linking enhancement was observed only at positions 8 and 19.

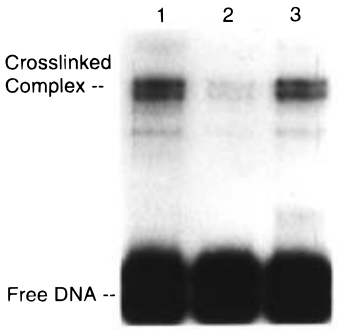

FIGURE 5: Confirmation of specific complex formation. The effects of specific and nonspecific DNA competitors on complex formation and cross-linking were analyzed on a 10% SDS−polyacrylamide gel. All the BrU-substituted DNA used for this experiment was modified at position 8. Lane 1, ^{32}P-labeled BrU DNA (2.0 μM) and 1.5 μM Tus; lane 2, ^{32}P-labeled BrU DNA (2.0 μM), 1.5 μM Tus, and 20 μM unlabeled wild-type DNA competitor; lane 3, ^{32}P-labeled BrU DNA (2.0 μM), 1.5 μM Tus, and 20 μM unlabeled nonspecific DNA competitor. All were irradiated for 30 min from 10 cm.

the portion of protein involved in the cross-link, the complex was subjected to extensive proteolysis using trypsin (Figure 6). Proteolysis of the complex produced a major fragment that migrated faster than the cross-linked complex but slower than the free DNA.

Primary Sequence of the Cross-Linked Peptide. Our early attempts to purify the fragment by extraction from the gel were successful, but the material once isolated was not sufficiently soluble for sequence analysis (data not shown). Blotting proved to be an effective means of sequencing the fragment attached to the DNA. The PVDF membrane was able to bind the complex with high affinity and allowed us to remove the low molecular weight contaminants which interfere with sequence analysis. The peptide was sequenced directly from the PVDF membrane, and an 18 amino acid sequence from residue 122 to 139 was identified (Table 2).

DISCUSSION

Proteins that bind DNA are stabilized in part by "hydrophobic interactions". In all protein−DNA complexes, the removal of the methyl groups of thymine at some positions destabilizes the interaction (Aiken & Gumport, 1991; Brennan *et al.*, 1986; Caruthers, 1980; Caruthers *et al.*, 1987; Mazzarelli *et al.*, 1992; Newman *et al.*, 1990). For the Tus−*TerB* complex, we have previously observed that six methyl groups in the major groove between positions 8 and 19 were critical for the Tus−*Ter* binding interaction (Duggan *et al.*, 1995). By incorporating bromodeoxyuridine and iododeoxyuridine at each of these sites, we investigated possible

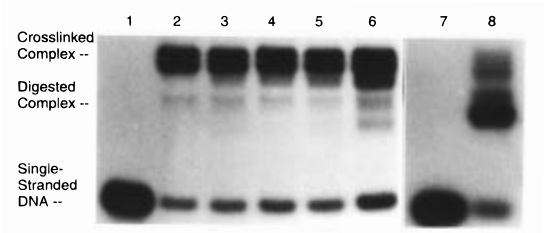

FIGURE 6: Electrophoretic assessment of trypsin proteolysis of the cross-linked complex on a 10% SDS−polyacrylamide gel: lane 1, single-stranded ^{32}P-labeled DNA reference; lane 2, ^{32}P-labeled complex prior to trypsin addition; lanes 3−6, progression of digestion after 1, 2, 4, and 12 h, respectively; lane 7, single-stranded ^{32}P-labeled DNA reference; lane 8, digestion nearing completion. After 48 h of digestion, the desired material migrated between the single-stranded DNA and undigested complex.

Table 2: Peptide Sequence of the Cross-Linked Fragment and Yield of Each Sequencing Cycle for the Amino Acid Detected

cycle	PTH amino acid	output (pmol)[a]	cycle	PTH amino acid	output (pmol)[a]
1	Thr	20.7	10	Glu	1.0
2	Thr	19.7	11	Ser	6.2
3	Phe	13.0	12	Glu	1.6
4	Glu	16.5	13	Leu	2.0
5	His	3.5	14	Pro	1.3
6	Ile	12.6	15	Thr	1.8
7	Val	9.0	16	Ala	1.5
8	Thr	8.8	17	Ala	4.8
9	Val	11.7	18	Arg	1.2

[a] All reported values listed in the output column are corrected using standards provided by Applied Biosystems Inc.

thermodynamic contributions to the interaction by the methyl group in the complex. These modified nucleotides are isosteric analogues of thymine because of the similarity of the van der Waals radii of the substituent groups at position 5 of the pyrimidine ring (Figure 1). The similarity in size prevents possible entropic effects of water that reduce the stability of the complex when uracil is used. However, insertion of a bromo or iodo group, with increased polarizability and electronegativity beyond that of a methyl group, will influence the interaction depending on the environment in which the halogen is placed. Consequently, a comparison of the responses by the various halogenated analogues in the complex provides information about the type of interaction used in the protein−DNA complex.

In all the cases in which we tested these analogues, we observed increased stability in relation to the uracil analogue (Duggan *et al.*, 1995). A comparison of thymine (methyl), bromodeoxyuridine, and iododeoxyuridine substituted *Ter* sites revealed two categories: those with favorable complex interactions and those with unfavorable interactions. The former is represented by the response when bromodeoxyuridine or iododeoxyuridine is inserted into positions 8, 14, and 19 where the stability of the protein−DNA complex is enhanced. These favorable changes are attributed to alterations of the local environment in which a polar group from the protein is now in close proximity to the halogenated base in the DNA. The magnitude of the free energy change seen with BrU at positions 8 and 14 is compatible with expectations for a dipole-induced dipole enhancement and is similar to increases observed for the *lac* repressor−operator interaction for similar substitutions (Goeddel *et al.*, 1978). The free energy change with BrU at position 19 of −0.87 kcal/mol is much larger than anticipated and might be explained by a more ionic environment which participates in the formation of the complex (Table 1).

To further test the validity of dipole-induced dipole interactions, we introduced iodouracil at these three positions and tested the resulting stability. Since the iodinated uracil has a larger dipole moment than the bromo counterpart, we anticipated that the presence of iodouracil would further stabilize the complex due to the enhanced dipole interaction. This was the case at positions 14 and 19. The iodouracil analogues at these two positions displayed half-lives greater than 2000 min, 10-fold above that measured for wild-type complex (Table 1). Consequently, we assume dipole-induced dipole interactions play a central role. The inability of the position 14 BrU to cross-link with the protein may arise because the amino acid adjacent to this site is not a suitable candidate for high-yield covalent attachment. Cross-linking is highly dependent on the type of amino acid involved in the reaction (Dietz & Koch, 1987, 1989). The third site at position 8 is believed to cross-link with a hydrophilic residue, clearly indicating that it too resides in a more polar environment (see below). We note that iodouracil positions at 14 and 19 will be useful for crystallographic analysis of the complex, since it allows the introduction of an electron-rich group needed for structural interpretation and at the same time increases the half-life of the complex.

Substitutions at positions 9, 12, and 16 with BrU displayed stability nearly equal to wild-type and reconstituted the majority of the binding lost by removing the methyl group when the uracil analogue was used. Destabilization of the protein−DNA complex occurred when iodouracil nucleotides were inserted at two positions: 12 and 16. Size effects are anticipated to play a role in the reduction of complex stability with IU at these two positions. The position 16 substitution had the largest effect, having a positive free energy change of 1.0 kcal/mol (Table 1). It is worthwhile noting that these two sites were affected dramatically by the exchange of uracil for thymine (∼2.0 kcal/mol), indicating that hydrophobic effects are of primary importance at these positions. Iodine could also lessen the favorable entropic contributions that accompany the formation of the complex in wild-type conditions.

These analyses reflect general trends of the data. Subtle conformational changes could enhance or reduce the stability of the complex. Halogen substitutions has been shown to alter base stacking (Sternglanz & Bugg, 1975), although that work uses totally substituted DNA and is probably not representative of singly substituted DNA. Changes that result from multiple effects will require deeper analysis of complex formation. However, it is now clear from our data that methyl groups contribute to the stability of this complex in different ways.

A useful feature of halogenated uracil derivatives is their ability to be photoactivatible with UV light, thereby cross-linking the DNA and protein. Because ultraviolet light is a "zero length" crosslinker, an amino acid residue would have to be extremely close to cross-link to an excited base. This provides a glimpse at the arrangement of the complex. Both the iodine and the bromine derivatives of uridine have been used for their increased photosensitivity to UV light (Allen *et al.*, 1991; Blatter *et al.*, 1992; Gott *et al.*, 1991; Lin & Riggs, 1974; Ogata & Gilbert, 1977; Simpson, 1979; Wick & Matthews, 1991; Willis *et al.*, 1993).

We have concentrated our efforts on the position 8 reaction and have isolated a tryptic fragment whose sequence constitutes the 18 amino acid segment from amino acid 122 to amino acid 139 of the Tus protein (see Table 2). The exact site of cross-linking is a matter of speculation at this time. However, the significant reduction in the yield of PTH-derivatized amino acids starting at cycle 10 (Table 2) implies that cross-linking occurred in the latter part of the helix. Cross-linking is dependent on the nature of the amino acid; certain sites could be ruled out as the cross-linking position. Dietz and Koch (1987, 1989) found that with BrU the oxidizable aromatic amino acids Tyr, Trp, and His, as well as cystine, are the most reactive. In general, polar groups on the proteins are more easily cross-linked than apolar groups (Wick & Matthews, 1991). This raises the possibility that cross-linking occurs at either Glu, Ser, or Glu of the sequenced peptide. Mutational analysis is required to address this possibility.

The region containing this sequence is predicted to be an amphiphilic α-helical segment based on helical net diagrams (Nedved *et al.*, 1994), and amphiphilic α-helix as a motif for DNA major groove binding has been demonstrated for other DNA binding proteins (Pabo & Sauer, 1992). This single helix cannot adequately account for all the methyl group interactions in the protein−DNA complex, since the protein helix would have to follow the curvature of the DNA one full turn, assuming a standard B-DNA helix. This problem is exemplified by considering the three methyl groups at positions 8, 14, and 19. The position 14 methyl group is situated on the DNA approximately 180° in relation to the other two methyl groups. Such a protein helical arrangement is highly unlikely and raises the possibility that either structural effects alter the stability of the complex or other portions of the protein are involved in binding. The former explanation would be inconsistent with our previous work (Duggan *et al.*, 1995). Using analogues, we have demonstrated that both the N7 and O6 of the G residue at position 13, located in the major groove, are essential for protein−DNA stability. Consequently, major groove chemical groups of the position 14 base pair, adjacent to this essential site, are positioned to interact directly with the protein, although indirect effects are not excluded. From all these considerations, a second recognition element within the protein is proposed.

Current work on determining the sequence of the peptide that is capable of cross-linking with position 19 will provide us with a more complete picture of DNA binding by the Tus protein. With the location of at least part of the Tus DNA binding site, efforts can now focus on genetic work. Tus mutants of this area can be used to clarify the mechanism of replication arrest, the nature of the Tus−*Ter* interaction, and possibly the differential binding to the multiple *Ter* sites.

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