Use of electrospray ionization mass spectrometry to study binding interactions between a replication terminator protein and DNA

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Abstract

Tus protein binds tightly to specific DNA sequences (*Ter*) on the *Escherichia coli* chromosome halting replication. We report here conditions for detecting the 1 : 1 Tus–*Ter* complex by electrospray ionization mass spectrometry (ESI-MS). ESI mass spectra of a mixture of Tus and nonspecific DNA showed ions predominantly from uncomplexed Tus protein, indicating that the Tus–*Ter* complex observed in the gas phase was the result of a specific interaction rather than nonspecific associations in the ionization source. The Tus–*Ter* complex was very stable using a spray solvent of 10 mM ammonium acetate at pH 8.0, and initial attempts to distinguish binding affinities of Tus and mutant Tus proteins for *Ter* DNA were unsuccessful. Increasing the ammonium acetate concentration in the electrospray solvent (800 mM at pH 8.0) increased the dissociation constants sufficiently such that relative orders of binding affinity for Tus and various mutant Tus proteins for various DNA sequences could be determined. These were in agreement with the dissociation constants determined in solution studies. A dissociation constant of 700×10^{-9} M for the binding of the mutant Tus protein A173T (where residue 173 is changed from alanine to threonine) to *Ter* DNA was estimated, compared with a value of $\leq 2 \times 10^{-9}$ M for Tus where A173 was unchanged. This is the first example in which ESI-MS has been used to compare binding affinities of a DNA-binding protein with mutant proteins for specific DNA recognition sequences. It was also possible to estimate the strength of the interaction between Tus and a DNA sequence (*TerH*) that had been identified by database searching.

Keywords: Tus; DNA replication; electrospray ionization mass spectrometry; noncovalent complex; dissociation constant

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Electrospray ionization mass spectrometry (ESI-MS) is now established as a powerful tool for analysis of the primary structure of biomolecules (Griffiths et al. 2001). More recently, this technique has been applied to the study of noncovalent biochemical complexes (Loo 1997). There are several technical difficulties to overcome to observe complexes involving macromolecules in the gas phase. For example, buffers used in solution studies of biochemical complexes are not usually volatile and therefore are not compatible with mass spectrometry. Furthermore, solvents used in ESI-MS typically contain an organic phase that could disrupt noncovalent complexes. Keeping this in mind, it is important to prepare the complex under conditions in which it maintains its native, folded state, and to use instrumental conditions such that it is ionized and transported to the mass analyzer intact. One of the most important criteria to be satisfied concerns whether noncovalent complexes observed

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in the gas phase reflect solution behavior or are the result of nonspecific associations in the ion source. As a consequence, ESI-MS of noncovalent complexes to date has been conducted on systems that have been well characterized in solution. Accumulation of information concerning a range of various binding partners prepared and analyzed under a range of conditions will shed light on the question of whether mass spectrometry can be used to study noncovalent interactions.

Interactions of proteins with nucleic acids are important in replication, repair, transcription, and translation. There have been fewer than 10 ESI-MS studies of noncovalent complexes of DNA with proteins and only a few of these involved complexes of intact proteins with double-stranded (ds)DNA (Cheng et al. 1996a; Potier et al. 1998; Craig et al. 1999). We have used ESI-MS to study the well-characterized interactions of *Escherichia coli* Tus protein (35,652 Daltons) with its DNA recognition sequence, *TerB*. Six termination sequences (*TerA-F*) have been identified on the *E. coli* chromosome, and each contains a consensus sequence that is ~20 bp long. Tus binds as a monomer to termination sequences, halting replication. Termination of replication shows polarity in that when Tus binds to the chromosome, it stops the replication fork moving in one direction but not the other. A key to this polarity is found in the asymmetry of the complex revealed in the X-ray crystal structure of Tus with a 16-bp *Ter* DNA (Kamada et al. 1996). Equilibrium dissociation constants (K_d) for the Tus–*Ter B* complex have been measured using gel mobility shift and filter binding assays (Gottlieb et al. 1992; Skokotas et al. 1994; Coskun-Ari and Hill 1997) and in surface plasmon resonance (SPR) experiments (Neylon et al. 2000). The binding is very tight: K_d values are 3.3×10^{-13} M in buffer at pH 7.5, containing 150 mM glutamate (Gottlieb et al. 1992), and 0.5×10^{-9} M in buffer at pH 7.6, containing 250 mM KCl (Neylon et al. 2000). In the latter study, a range of KCl concentrations was investigated, and extrapolation of data to KCl = 150 mM gave K_d ~1×10^{-12} M.

The availability of the X-ray crystal structure (Kamada et al. 1996), combined with in vivo and in vitro binding studies of native Tus compared with mutant proteins has enabled analysis of the relative contributions of various polar and hydrophobic interactions to binding. In addition, variant *Ter* sequences have been studied (Coskun-Ari and Hill 1997). In the Tus–*Ter* complex, the DNA lies in a positively charged cleft between N- and C-terminal domains that are joined by interdomain β strands (Kamada et al. 1996). Fourteen Tus residues make sequence-specific contacts with *Ter* DNA, and there are numerous polar contacts between Tus and the phosphate backbone. Importantly, on the side of the complex that allows the replication fork to proceed, one DNA strand makes extensive contact with Tus, whereas the other strand is predominantly exposed to solvent. In contrast, on the side of the complex in which replication is halted, Tus makes extensive contacts with both DNA strands (Kamada et al. 1996; Neylon et al. 2000).

In the cleft, the side chain of Ala 173 is involved in a hydrophobic interaction with the methyl group of a thymine base. When this alanine residue is changed to the more bulky threonine (A173T), Tus binds ~4000-fold less tightly to *TerB* and is unable to halt replication in vivo (Skokotas et al. 1994; Neylon et al. 2000). Arg 198 lies just outside the core DNA-binding region on the side of the complex in which replication is halted and makes sequence-specific contacts with DNA (Kamada et al. 1996). Investigations of the interactions of these and other Tus mutants with *Ter* and nonspecific DNA and the effect of salt concentration on the binding have led to the proposal of a binding mechanism, wherein an initial nonspecific binding event involving interactions of Arg 198 and other basic residues with DNA is followed by sequence recognition by residues including Lys 89. Strong sequence specific contacts (e.g., with Ala 173) then can be made concomitant with a conformational change of Tus (Neylon et al. 2000).

Several ESI-MS studies of noncovalent complexes of DNA with proteins have been able to distinguish between binding of the protein with specific or nonspecific DNA (Cheng et al. 1996b; Potier et al. 1998), or in the case of a DNA repair protein, with damaged or undamaged DNA (Xu et al. 1999). In work reported here, we have studied the Tus–*Ter* interaction and describe the first use of ESI-MS to compare the relative strengths of binding of native and mutant proteins with specific DNA sequences. It was necessary in binding studies of the Tus–*Ter* complex using SPR to increase salt concentration to allow measurement of association and dissociation rates (and K_d). Similarly, in this work we needed to weaken the binding to distinguish between Tus and Tus mutants in complexes with *Ter* DNA; we used 800 mM ammonium acetate at pH 8.0, as the solvent in ESI-MS. In previous ESI-MS studies of DNA–protein complexes, low concentrations (usually 10 mM) of ammonium acetate or bicarbonate have been used. The use of an ESI time-of-flight mass spectrometer with a Z-spray probe has made the use of high salt possible. Conditions under which proteins are fully folded are more likely to be found if a wide range of salt conditions (10–2200 mM) are tested. The use of higher salt concentrations also decreases the chance that nonspecific gas phase associations of molecules will be observed (Sannes-Lowery et al. 2000).

Results and Discussion

Figure 1 shows the sequences of DNA strands used in this work. All except the nonspecific DNA were 21 nucleotides long, and *TerB* had the same sequence used in our earlier SPR studies of Tus–*Ter* complexes (Neylon et al. 2000). In a previous study of the effect of base pair substitutions in *Ter* DNA on the Tus–*Ter* complex, Coskun-Ari and Hill

	Mass (Da)	Sequence
TerB	12846.6	5′ A T A A G T A T G T T G T A A C T A A A G
TerH	12848.5	5′ G A T C • • • • • • • • • • • • • • T C T
posn5 *TerB*	12846.6	5′ • • • • C • • • • • • • • • • • • • • • •
posn7 *TerB*	12847.6	5′ • • • • • • G • • • • • • • • • • • • • •
posn10 *TerB*	12847.6	5′ • • • • • • • • • G • • • • • • • • • • •
non-specific	14701.6	5′ C T T A T G A G C T T A T A A G C T C A T A A G

Fig. 1. Sequences of dsDNAs used in this work; only one strand of each is shown. The dots indicate that the base in that position is the same as in the *TerB* sequence. The masses are those for double-stranded DNA, i.e., the strand shown in addition to its complement. Da, Dalton.

(1997) used 33-bp oligonucleotides. The 21-bp oligonucleotides used in the present work contain the core sequence necessary for binding to Tus. The sequences termed position 5, 7, and 10 substitutions of *TerB* are the same variations termed position 6, 8, and 11 substitutions in the study by Coskun-Ari and Hill (1997). These sequences are here referred to as posn5*TerB*, posn7*TerB*, and posn10*TerB*, respectively. *TerH* was identified as a possible strong binding site for Tus by searching the *E. coli* genomic DNA sequence, but has not been examined previously by experiment (Coskun-Ari and Hill 1997). The nonspecific DNA is a self-complementary 24-bp sequence.

As a first step in ESI-MS of noncovalent DNA–protein complexes, it is important in both the preparation of the complex and in choosing the spray solvent to use solution conditions in which the protein is in its native, folded conformation. Figure 2 shows ESI mass spectra of Tus that had been dialyzed into 10 mM ammonium acetate (NH_4OAc) over the pH range 5.0 to 8.0 and infused directly into the ionization source. The ESI mass spectrum of Tus at pH 5.0

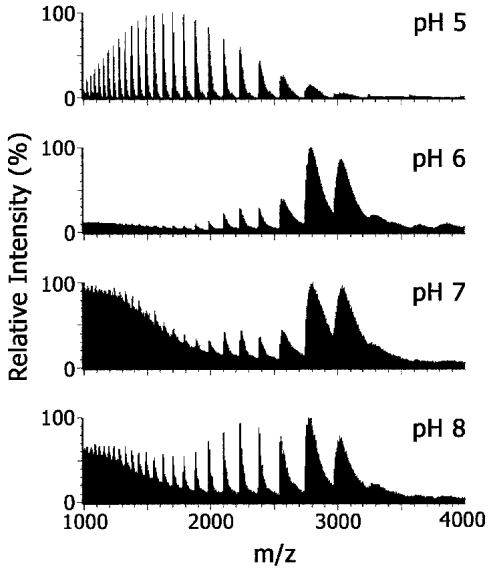

Fig. 2. ESI mass spectra of Tus (10 μM) in 10 mM NH_4OAc at pH values of 5.0, 6.0, 7.0, and 8.0. Instrumental conditions were as described in Materials and Methods except that the desolvation temperature was 60°C.

showed numerous ions with $[M + 25H]^{25+}$ (m/z 1427.1) the most abundant. At higher pH values, the charge distribution is markedly different with ions observed at higher values of m/z with $[M + 13H]^{13+}$ (m/z 2743.5) the most abundant. This change in the charge envelope generally is observed on acidification of proteins and has been explained in terms of unfolding of the protein leading to exposure of a greater number of basic residues (Konermann and Douglas 1998; Jarrold 1999). There is evidence from nuclear magnetic resonance spectroscopy that Tus is fully folded at pH 8.0, but unfolds at pH <6.0 (G. Otting and N.E. Dixon, unpubl.). At pH ≥6.0, the ions are very broad (m/z ~250 at half height), indicating incomplete desolvation of the protein under these conditions. This observation suggests that water/salt molecules trapped in the folded structure are released as the protein unfolds.

In these preliminary ESI-MS experiments, low desolvation temperatures (60°C) were used. In previous work, low temperatures had enabled detection of noncovalent complexes of intercalators with double-stranded DNA (Kapur et al. 1999). These conditions gave spectra of the Tus–*Ter* complex in which the peaks were broad (Fig. 2, pH 6.0 to 8.0), and mass accuracy therefore was low. An experiment was conducted to determine the effect of desolvation temperature on ESI mass spectra of a 1 : 1 Tus–*TerB* complex in 10 mM NH_4OAc at pH 8.0, over the range 60 to 240°C (data not shown). As expected, increasing desolvation temperature resulted in sharper peaks in the ESI mass spectra. At 240°C, the complex remained intact and sharp peaks were obtained with the highest signal-to-noise ratio. The width-at-half-height of ions from the complex was m/z of ~5 (m/z ~40 at base). The signal-to-noise ratio was improved by the use of an elevated pressure of argon in the collision cell and a collision energy of ~20 eV. This presumably reduces the energy spread of the ions entering the time-of-flight analyzer.

Figure 3 shows ESI mass spectra of mixtures of Tus with nonspecific (Fig. 3A), *TerB* (Fig. 3B), and posn10*TerB* (Fig. 3C) DNA in 20 mM ammonium acetate at pH 8.0, under optimized instrumental conditions. For samples containing *TerB* and posn10*TerB*, the only significant ions in the spectra were from 1 : 1 Tus–DNA complexes ($[M + 14H]^{14+}$, $[M + 15H]^{15+}$, and $[M + 16H]^{16+}$ ions, at

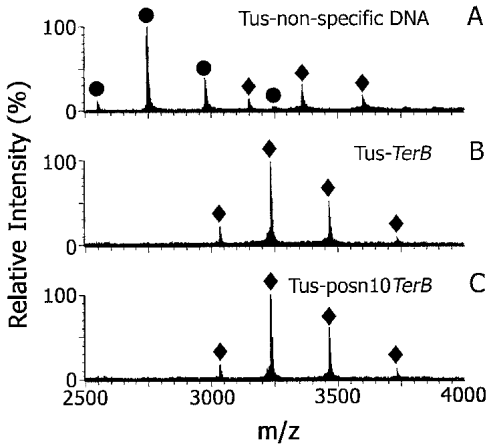

Fig. 3. ESI mass spectra of Tus–dsDNA complexes (10 μM) in 20 mM NH_4OAc at pH 8.0. (*A*) Tus-nonspecific DNA, (*B*) Tus–*TerB*, (*C*) Tus–posn10*TerB*. (circles) Ions from free Tus protein; (diamonds) ions from Tus–dsDNA complexes.

m/z 3465.5, m/z 3234.5, and m/z 3032.4, respectively; see Table 1). Significantly, in the spectrum of Tus with nonspecific DNA recorded under the same conditions, the predominant ions were from free Tus (Fig. 3A). The observation of the complex of Tus with *TerB* DNA but not with nonspecific DNA suggests that the complex observed in the gas phase is not the result of nonspecific associations in the ionization source. There is also a small amount of Tus-nonspecific DNA complex evident in the spectrum. Weak binding to nonspecific DNA also was observed in solution studies with Tus (Coskun-Ari and Hill 1997; Neylon et al. 2000) and in studies on other DNA-binding proteins (Ha et al. 1992). Furthermore, the first step in the proposed binding mechanism of Tus to *TerB* involves nonspecific electrostatic interactions of positively charged residues of Tus with the phosphate backbone of DNA (Neylon et al. 2000).

The posn10*TerB* (a T · A base pair changed to G · C) was shown using filter binding assays to bind to Tus with an equilibrium dissociation constant (K_{obs}) of 1204×10^{-13} M compared with the value for native *TerB* of 9×10^{-13} M. In addition, the in vivo replication arrest activity of Tus bound at a native *TerB* site was 95%, compared with only 2% efficiency for this substituted DNA (Coskun-Ari and Hill 1997). These observations were explained in terms of removal of a hydrophobic interaction between Val 234 and the thymidine in the T · A base pair normally present at position 10 of *TerB* (Coskun-Ari and Hill 1997). Under the conditions of this ESI-MS experiment, we could not distinguish between binding of Tus with *TerB* (Fig. 3B) or posn10*TerB* (Fig. 3C). This is in accord with the relative concentrations of posn10*TerB*–Tus complex ($Tus_{complex}$) and free Tus (Tus_{free}) in solution, calculated using the value of K_{obs} (1204×10^{-13} M, above): [Tus_{free} = 0.03 μM, $Tus_{complex}$ = 9.97 μM. Thus, if binding in the gas phase under our experimental conditions were at least as tight as in solution, then ions from free Tus would not be readily observable in the ESI mass spectrum. This also assumes that the response factors of Tus and Tus–*TerB* complex are comparable. The response factor is used to describe the efficiency with which gas phase ions are formed and detected in the mass spectrometer. Certainly, given the lower net charge on the complex than on free Tus, it seems unlikely that in positive ion ESI mass spectra the response factor would be lower for free Tus than for the DNA–Tus complex.

Similarly, in ESI mass spectra of complexes of unmodified Tus with posn5*TerB*, posn7*TerB*, or with *TerH*, there were no ions from free Tus or DNA. Furthermore, mixtures of *TerB* with Tus, the A173T mutant of Tus, or N-terminal $(His)_6$-tagged Tus (his_6Tus) all gave ESI mass spectra in which only ions from complexes and not from free binding partners were observed. This suggests that under the conditions of these experiments, all these complexes were too

Table 1. *Calculated values of* m/z *for ions observed in ESI mass spectra*

Protein	Complex[a]	$[M + 11H]^{11+}$	$[M + 12H]^{12+}$	$[M + 13H]^{13+}$	$[M + 14H]^{14+}$	$[M + 15H]^{15+}$	$[M + 16H]^{16+}$
Native Tus	Free[b]	3242.1	2972.0	2743.5	2547.6	2377.8	2229.3
(35 652 Da)	*TerB*[c]	4410.0	4042.5	3731.7	3465.2	3234.2	3032.2
	Nonspecific	4578.6	4197.1	3874.4	3597.7	3357.9	3148.1
His_6Tus	Free[b]	3340.7	3062.4	2826.9	2625.1	2450.1	2297.1
(36 737 Da)	*TerB*[c]	4508.6	4133.0	3815.2	3542.7	3306.6	3100.0
	Nonspecific	4677.3	4287.6	3957.8	3675.2	3430.3	3215.9
R198A	Free[b]	3333.0	3055.3	2820.4	2619.0	2444.5	2291.8
(36 652 Da)	*TerB*[c]	4500.9	4125.9	3808.6	3536.6	3300.9	3094.7
	Nonspecific	4669.5	4280.5	3951.3	3669.1	3424.6	3210.6
A173T	Free[b]	3343.5	3064.9	2829.2	2627.2	2452.1	2298.9
(36 767 Da)	*TerB*[c]	4511.3	4135.5	3817.4	3544.8	3308.6	3101.8
	Nonspecific	4680.0	4290.1	3960.1	3677.3	3432.3	3217.8

[a] This column shows the DNA present in the complex.
[b] "Free" refers to the protein in the absence of DNA.
[c] The *m/z* values for complexes of Tus proteins with posn5,7,10*TerB* are not given since they are the same within 0.2 Da as the complexes with *TerB* DNA.

tightly bound to enable observation of free binding partners. In SPR studies, the equilibrium dissociation constants for Tus and his_6Tus were indistinguishable (Neylon et al. 2000). In subsequent experiments (below), his_6Tus (rather than unmodified Tus) was used to enable more direct comparisons with mutant Tus proteins (A173T and R198A), which both carried a hexahistidine tag.

In SPR experiments with Tus or his_6Tus, it was possible to determine equilibrium dissociation constants only when the binding interaction was weakened by increasing the KCl concentration (Neylon et al. 2000). Similarly, we reasoned that increasing the ionic strength of the spray solvent would allow distinction between complexes of Tus and mutants with DNA in ESI mass spectra. Most ESI-MS studies of noncovalent complexes have used 10–50 mM ammonium acetate, with one report of a mass spectrum of dsDNA obtained in 150 mM ammonium acetate at pH 7.0 (Hofstadler and Griffey 2001). An experiment was conducted in which ESI-MS spectra of Tus–*Ter* (10 μM) complexes were acquired over a range of ammonium acetate concentrations from 10 to 2200 mM at pH 8.0. Figure 4 shows the effect of increasing NH_4OAc concentration on the his_6Tus–*TerB* and A173T–*TerB* complexes. Ions were observed at m/z 3543.2, 3307.0, and 3100.4 for his_6Tus–*TerB*, and at m/z 3545.5, 3309.2, and 3102.4 for A173T–*TerB*. The A173T–*TerB* complex is almost completely dissociated when the solvent is 800 mM NH_4OAc, whereas the ESI mass spectrum of the his_6Tus–*TerB* complex at this salt concentration shows ions only from the complex. The latter complex is ~50% dissociated at NH_4OAc concentration = 1400 mM at pH 8.0 and is not completely dissociated until NH_4OAc concentration ≥2200 mM.

In all experiments, as ions corresponding to free Tus increased, ions appeared that corresponded to both single strands and ds*Ter* DNA (see electronic supplemental material). These additional ions were at m/z 2119.7 and 1590.4 ($[M + 3H]^{3+}$ and $[M + 4H]^{4+}$ of one *Ter* strand), at m/z 1624.7 ($[M + 4H]^{4+}$ of the other *Ter* strand), and at m/z 2142.6 and 1836.9 ($[M + 6H]^{6+}$ and $[M + 7H]^{7+}$ of ds*Ter*). The appearance of the DNA as single strands when it dissociates from Tus is expected based on other work in this laboratory showing that dsDNA denatures as the desolvation temperature is increased above 60°C. It seems likely that the response factor for DNA would be different from that for free Tus protein or the Tus–*Ter* complexes. Therefore, we have compared intensities of ions from free Tus with intensities of ions from complex in the following experiments.

An experiment was conducted over the same range of salt concentrations used to obtain the data in Figure 4, but comparing Tus–*TerB* with his_6Tus–*TerB*, A173T–*TerB*, and R198A–*TerB*. No significant differences could be detected between ESI mass spectra of Tus–*TerB* and his_6Tus–*TerB* at any salt concentration in the range 10 to 2200 mM (data not shown). This is in agreement with the SPR studies (Neylon et al. 2000). Figure 5 compares the complexes his_6Tus–*TerB*, A173T–*TerB*, and R198A–*TerB*. The data were obtained by summing the intensities of all ions from the com-

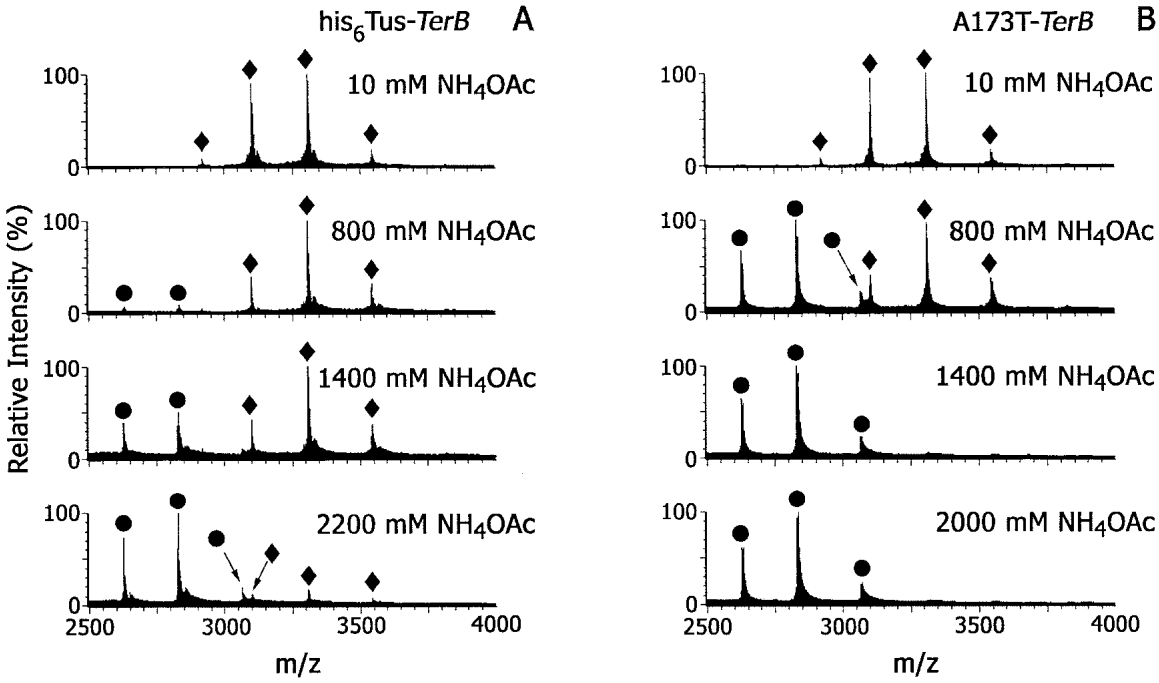

Fig. 4. ESI mass spectra of the Tus–dsDNA complexes (10 μM). (*A*) his_6Tus–*TerB* and (*B*) A173T–*TerB* at NH_4OAc concentration = 10–2200 mM. (circles) Ions from free protein; (diamonds) ions from protein–dsDNA complex.

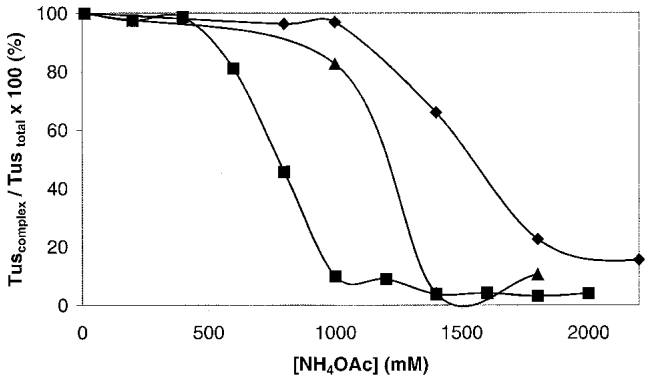

Fig. 5. Stability of complexes of his$_6$Tus and Tus mutants with *TerB*. The data show the decreasing amounts of Tus (or mutants) in the complex with dsDNA (Tus$_{complex}$) as a percentage of the total amount of Tus (Tus$_{total}$), as a function of NH_4OAc concentration. These values were determined by summing the intensities of all ions from Tus$_{free}$ and all ions from Tus$_{complex}$. Tus$_{free}$ + Tus$_{complex}$ = Tus$_{total}$. (diamonds) his$_6$Tus–*TerB*, (triangles) R198A–*TerB*, (squares) A173T–*TerB*.

plex (Tus$_{complex}$) and from free Tus (Tus$_{free}$) and expressing each as a percentage of Tus$_{total}$ (Tus$_{total}$ = Tus$_{free}$ + Tus$_{complex}$; data shown for Tus$_{complex}$/Tus$_{total}$). The amount of each of the complexes decreased with increasing NH_4OAc concentration. The relative order of binding affinities can be determined by comparing the NH_4OAc concentration at which each complex is 50% dissociated: his$_6$Tus > R198A > A173T. The solution K_d values measured in 250 mM KCl using SPR for the his$_6$Tus–*TerB*, R198A–*TerB*, and A173T–*TerB* complexes were 0.5×10^{-9}, 130×10^{-9}, and 2000×10^{-9} M, respectively (Neylon et al. 2000). The present data are in very reasonable agreement.

Figure 6 shows data for the complexes of *TerB* and posn10*TerB* with each of his$_6$Tus and A173T. The order of stability of these complexes in increasing ammonium acetate concentrations is his$_6$Tus–*TerB* > his$_6$Tus–posn10*TerB* > A173T–*TerB* > A173T–posn10*TerB*. The dissociation constants of Tus–*TerB* and Tus–posn10*TerB* complexes are reported to be 9×10^{-13} and 1204×10^{-13} M, respectively (Coskun-Ari and Hill 1997). The A173T–*TerB* complex has not been compared previously with the A173T–posn10*TerB* complex. It might be expected that changing both an important sequence specific contact in the protein (A173T) concomitant with changing a base in the DNA causes the binding to be less avid than changing either one of the binding partners on its own.

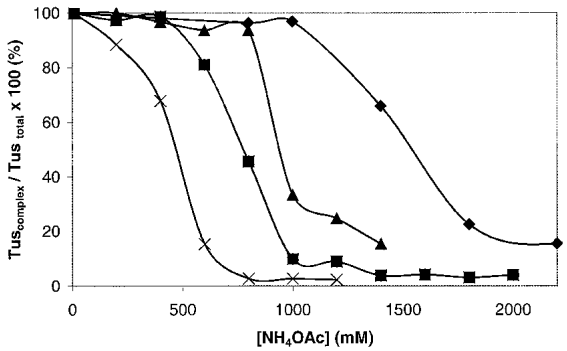

Fig. 6. Stability of complexes of his$_6$Tus and A173T with *TerB* and posn10*TerB*. The data show the decreasing amounts of his$_6$Tus or A173T in the complex with dsDNA (Tus$_{complex}$) as a percentage of the total amount of Tus (Tus$_{total}$), as a function of NH_4OAc concentration. (diamonds) his$_6$Tus–*TerB*, (triangles) his$_6$Tus–posn10*TerB*, (squares) A173T–*TerB*, (multiplication symbols) A173T–posn10*TerB*.

These relative binding affinities were confirmed in competition experiments conducted in 800 mM ammonium acetate at pH 8.0. ESI mass spectra of 1 : 1 : 1 mixtures (10 μM each) of various protein and DNA samples were acquired. For example, to determine whether his$_6$Tus, A173T, or R198A bind more tightly to *TerB*, the following mixtures were set up: his$_6$Tus : A173T : *TerB*, his$_6$Tus : R198A : *TerB*, and R198A : A173T : *TerB*. Table 2 summarizes the competition mixtures that were used and shows the complexes and free binding partners that were observed in the spectra. Under the conditions of these experiments, the K_d values of the complexes compared here were sufficiently disparate that each ESI mass spectrum showed ions from only one DNA–protein complex together with ions from the free protein not involved in the complex. Analysis of the spectra summarized in Table 2, part A, shows clearly that the proteins bind to *TerB* in the order his$_6$Tus > R198A > A173T. This is consistent with data in Figure 5 and K_d values measured in SPR experiments. Analysis of the spectra summarized in Table 2, part B, show that the proteins bind to posn10*TerB* in the same order.

The relative binding affinities of his$_6$Tus for *TerB* DNA substituted at positions 5, 7, and 10 and for *TerH* DNA could not be determined in competition experiments because the difference in mass between an A · T and a G · C base pair is only 1 Dalton, and the resolution in the ESI mass spectra was not sufficient to distinguish between complexes involving these different DNA sequences. The relative stabilities of these complexes were tested in NH_4OAc in the same way as experiments shown in Figures 5 and 6. The data in Figure 7 show that the modified *Ter* sequences bind to his$_6$Tus in the order *TerB* > posn5*TerB* > *TerH* > posn7*TerB* ~ posn10*TerB*. These results are consistent with values of K_{obs} measured in solution of 9×10^{-13}, 16×10^{-13}, 139×10^{-13}, and 1204×10^{-13} M for *TerB*, posn5*TerB*, posn7*TerB*, and posn10*TerB*, respectively (Coskun-Ari and Hill 1997). It is difficult to determine unequivocally from these data the position in the binding order of posn10*TerB* relative to posn7*TerB*. In posn5*TerB*, a G · C has been changed to a C · G base pair. This removes an interaction between Arg 198 of Tus and the N-3 atom of the guanine residue. In posn7*TerB*, an A · T base pair has been changed to G · C, removing interactions of the O-2 of the thymine base with Lys 89, and a major

Table 2. *Results of competition experiments*

	Ions observed in the ESI-mass spectrum					
Mixture components	His_6Tus	A173T	R198A	His_6Tus-TerB	A173T-TerB	R198A-TerB
A						
His_6Tus: A173T: TerB	×	√	—	√	×	—
His_6Tus: R198A: TerB	×	—	√	√	—	×
R198A: A173T: TerB	—	√	×	—	×	√
B	His_6Tus	A173T	R198A	His_6Tus-posn10TerB	A173T-posn10TerB	R198A-posn10TerB
His_6Tus: A173T: posn10TerB	×	√	—	√	×	—
His_6Tus: R198A: posn10TerB	×	—	√	√	—	×
R198A: A173T: posn10TerB	—	√	×	—	×	√

Mixture components were present in 1:1:1 molar ratios in 800 mM NH_4OAc, pH 8. In the ESI mass spectra of these mixtures, only one complex was observed: (×) ions not observed; (√) ions observed; (—) not a mixture component in this experiment.

groove interaction of the thymine methyl group with Thr 139 (Kamada et al. 1996; Coskun-Ari and Hill 1997). The *TerH* site was identified by database searching (Coskun-Ari and Hill 1997). On the basis of *Ter* base pair substitution studies, it was proposed to be a moderately strong site, but this was not confirmed by experiment. The present results suggest that this is true.

Collision-induced dissociation (CID) experiments (either in the source or collision cell) have been used in ESI-MS studies of noncovalent complexes as a measure of the stability of the binding interaction (Schwartz et al. 1995; Wan et al. 2000). In a study of the interactions of the Trp repressor with its consensus operator DNA sequence, an increase in cone voltage from 100 to 200 V resulted in only partial dissociation of the complex with the appearance of low abundance ions at values of m/z corresponding to complexes with a mass ~150 Daltons lower than ions from intact complex. These ions were thought to arise from depurination of DNA (Potier et al. 1998). In our early experiments, attempts to disrupt the Tus–*TerB* or A173T–*TerB* complexes prepared and analyzed in 10 mM ammonium acetate at pH 8.0, by increasing the cone voltage from 50 to 100 V were unsuccessful. Furthermore, none of the complexes of Tus with substituted *Ter* sequences (Fig. 1) or of A173T with any of these DNA sequences was dissociated under these conditions. This experiment was repeated using 800 mM ammonium acetate at pH 8.0 as solvent. Figure 8 shows ESI mass spectra of the his_6Tus–*TerB* (Fig. 8A) and A173T–*TerB* (Fig. 8B) complexes, as a function of cone voltage. At 25 V, A173T–*TerB* is ~50% dissociated in this solvent, and the complex further dissociates as the cone voltage is increased to 100 V. In contrast, the his_6Tus–*TerB* complex remains intact up to 100 V. This CID experiment confirms the data above which show that his_6Tus binds the *TerB* sequence more tightly than the mutant A173T. The spectrum of the his_6Tus–*TerB* complex (Fig. 8A) obtained using a cone voltage of 100 V shows that ions from the complex are shifted to lower m/z values. Tus protein alone was stable at this cone voltage, suggesting that as in experiments with the Trp repressor (Potier et al. 1998) there may have been some fragmentation of DNA in the complex. In a separate experiment, resolution was sufficient to enable observation of an ion corresponding to a mass loss of ~134 Daltons (data not shown), consistent with loss of adenine.

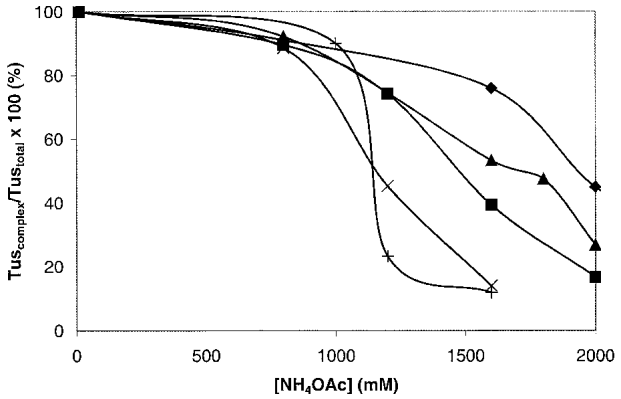

Fig. 7. Stability of complexes of his_6Tus with the variant *Ter* DNAs. The data show the decreasing amounts of his_6Tus in the complex with dsDNA ($Tus_{complex}$) as a percentage of the total amount of Tus (Tus_{total}), as a function of NH_4OAc concentration. (diamonds) his_6Tus–*TerB*, (triangles) his_6Tus–posn5*Ter*, (squares) his_6Tus–*TerH*, (multiplication symbols) his_6Tus–posn7*TerB*, (plus signs) his_6Tus–posn10*TerB*.

The Tus–*Ter* interaction involves many electrostatic contacts, therefore increasing salt concentration markedly decreases the stability of the Tus–*TerB* complex in solution studies. This general observation has been confirmed in the present ESI mass spectra. The solvent used in the SPR study was 50 mM Tris-HCl at pH 7.5, 0.1 mM EDTA, 0.1 mM DTT, 0.005% Nonidet P-20, 250 mM KCl, which is markedly different from ammonium acetate used for ESI-MS. To comment further on whether the solution and gas phase complexes are the same, values of K_d need to be measured by titrating Tus with *TerB* and comparing these values with those determined in solution at similar ionic strength. The

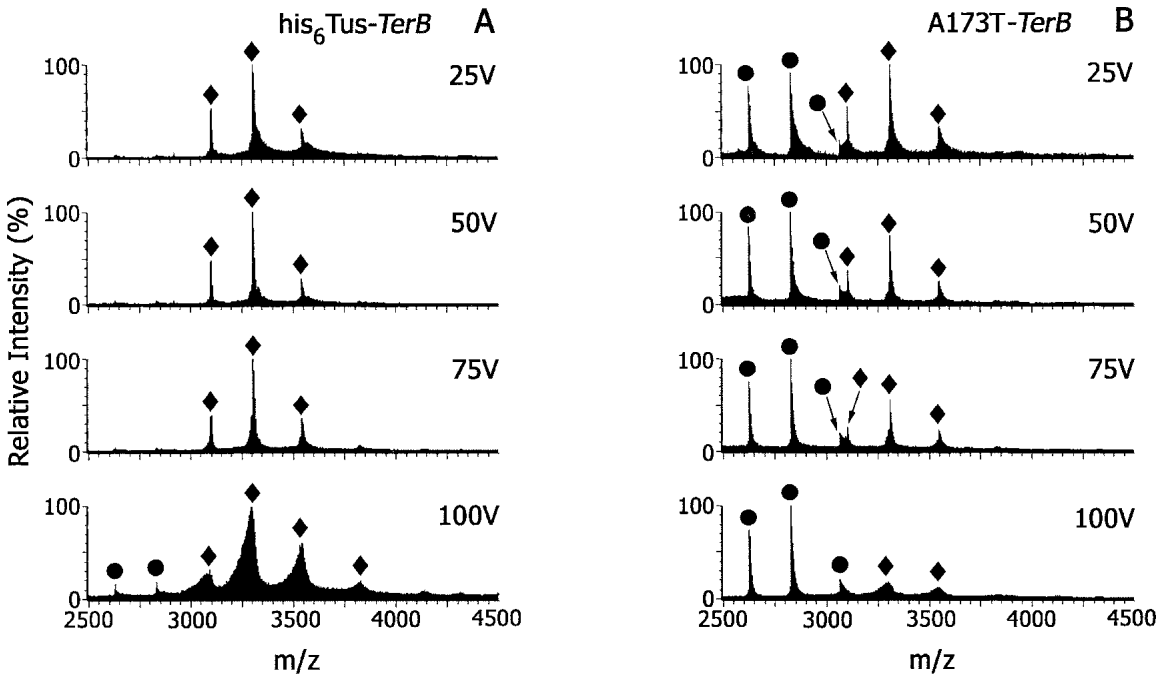

Fig. 8. The effect of increasing cone voltage on ESI mass spectra of (*A*) his_6Tus–*TerB* and (*B*) A173T–*TerB*, each 10 μM in 800 mM NH_4OAc at pH 8.0. (circles) Ions from free protein; (diamonds) ions from protein–dsDNA complex.

delineation of solvent and ESI-MS instrumental conditions that allow discrimination among the relative stabilities of various complexes is a starting point for being able to measure K_d values by ESI-MS.

ESI-MS has some advantages over filter binding and gel retardation assays for study of protein–DNA interactions in that these techniques necessarily require separation of bound and free mixture components for analysis and this may perturb the equilibrium position (Hagmar et al.1995). However, there are also complicating issues in determination of K_d values by ESI-MS. The first, alluded to above, is that the relative intensities of gas phase ions from free binding partners and complex may not correspond to the relative amounts of these species in solution if the response factors are markedly different. Response factors include the relative ionization efficiencies of solution components, but their determination is not a simple matter of predicting ions based on solution pK_a values (Wang and Cole 1994; Constantopoulos et al. 1999; Cech and Enke 2000). The relative response factors for Tus and Tus–*Ter* complexes were determined by titrating A173T into a solution containing 1 : 1 his_6Tus–*TerB* complex in 800 mM NH_4OAc at pH 8.0. Under these conditions, the his_6Tus–*TerB* complex is stable and A173T is not expected to bind *TerB* (Fig. 4). The total concentration of all Tus (Tus_{total}; A173T + his_6Tus in the complex) was maintained at 10 μM. A plot of the ratio of the intensities of ions from A173T (Tus_{free}) to the intensities of ions from the complex ($Tus_{complex}$) in the ESI mass spectrum against the ratios of free A173T and his_6Tus–*TerB* complex added to the solution showed the response factors of free Tus and the complex to be the same within experimental error (see electronic supplemental material). Note that relative orders of binding obtained by determining the NH_4OAc concentration at which complexes dissociate (Figs. 4–7) are independent of response factors of free Tus and complex.

A second complication is that electrostatic interactions are thought to be strengthened in vacuo. Therefore, the relative contributions of electrostatic interactions, hydrogen bonding, and hydrophobic and van der Waals interactions to the free energy of binding will influence stabilities of noncovalent complexes in the ESI source (Loo 1997). An example of where this may have an impact on values of dissociation constants estimated in the gas phase would be in comparisons of Tus with R198A. Arg 198 is involved in interactions with the negatively charged DNA backbone. If this and other electrostatic interactions are strengthened in the mass spectrometer, then the difference in the strength of *TerB* binding by Tus and R198A would be greater in the gas phase than in solution.

Scatchard plots (for measuring K_d) determined in the gas phase have been generated for noncovalent complexes of vancomycin antibiotics with tripeptides (Lim et al. 1995), and for complexes of aminoglycoside antibiotics with RNA (Sannes-Lowery et al. 2000), and were in reasonable agreement with solution data. We attempted to titrate Tus proteins with *TerB* DNA but encountered a small amount of precipitation of a component of the solution at stoichiom-

etries of *TerB* : Tus <1 : 1. Therefore, to estimate values of the K_d of Tus–*TerB* complexes, we collected ESI-MS spectra on serial twofold dilutions of the 1 : 1 complexes (over the range 0.23–15 μM in 800 mM ammonium acetate at pH 8.0). The results are presented in Figure 9. For the his_6Tus and R198A complexes, there was no change in the relative amounts of complex and free Tus as the complexes were diluted (Fig. 9). However, for the A173T–*TerB* complex, dilution gave rise to changes in concentrations of these species that suggest that under the conditions of this experiment, K_d for the A173T–*TerB* complex is $\sim 700 \times 10^{-9}$ M (Fig. 9). For his_6Tus–*TerB* and R198A–*TerB*, inability to observe an effect of dilution on relative amounts of free protein and complex implies that values of K_d of these complexes are $\leq 2 \times 10^{-9}$ M, based on the assumption that ions from free Tus can be observed once their total intensity (Tus_{free}) is ~6% of that of all Tus ions (Tus_{total}) in the ESI mass spectrum. For comparison, the solution K_d values measured in 250 mM KCl using SPR for the A173T–*TerB,* his_6Tus–*TerB*, and R198A–*TerB* complexes are 2000×10^{-9}, 0.5×10^{-9}, and 130×10^{-9} M, respectively (Neylon et al. 2000).

One of the primary experimental considerations in gas phase detection of noncovalent complexes of biological molecules is choice of conditions that are sufficiently gentle to maintain the integrity of the complex. In our study of the Tus–*TerB* interaction, we were able to observe ESI mass spectra of the complex with high sensitivity: down to 0.23 μM (11.5 pmole in 50 μL). The specificity of this interaction was shown by the inability to observe significant amounts of a complex with nonspecific DNA under conditions in which the Tus–*Ter* complexes were very stable. To distinguish subtle changes in dissociation constants using ESI-MS, it was necessary to weaken the binding by increasing the NH_4OAc concentration in the spray solvent. The relative orders of binding affinity determined were in reasonable agreement with solution studies, suggesting that the complexes observed in ESI mass spectra are representative of those in solution. This confirms that a specific interaction was being observed. Once conditions under which titrations of Tus with *TerB* can be conducted without precipitation of mixture components, it will be possible to determine dissociation constants and to make more direct comparisons with values measured in solution.

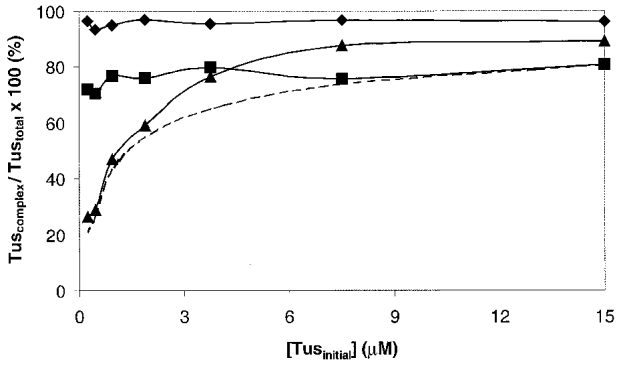

Fig. 9. The data show the amounts of Tus (or mutants) in the complex with *TerB* in 800 mM NH_4OAc at pH 8.0 ($Tus_{complex}$), as a percentage of the total amount of Tus (Tus_{total}) plotted against $[Tus]_{initial}$. The intensities of all ions in the complex were summed giving $Tus_{complex}$. The intensities of all ions from free Tus (Tus_{free}) and from the complex ($Tus_{complex}$) were summed to give Tus_{total}. (diamonds) his_6Tus–*TerB*, (squares) R198A–*TerB*, (triangles) A173T–*TerB*. (dashed line) The calculated curve for dissociation constant = 700×10^{-9} M.

The question as to the validity of dissociation constants measured in the gas phase has been addressed (Lim et al. 1995; Sannes-Lowery et al. 2000). The ionization process itself may perturb the structure of the complex and therefore the equilibrium position. The electrospray ionization process requires desolvation of charged droplets. In DNA–protein interactions, water molecules may have an integral structural role, for example, in forming H-bonds within and between binding partners (Schwabe 1997). The loss of water from the complex may have important implications for comparisons between the gas phase and solution. In the Tus–*TerB* complex, water molecules are involved in H-bonding between the DNA and protein (Kamada et al. 1996). Once DNA–protein contacts are formed in solution, whether any effects of instrumental conditions will be observed depends on the difference between the time required for a conformational change of the complex and the time between desolvation and detection by the mass analyzer. The extent of effects caused by transferal to the gas phase will depend on the nature of the interactions holding the complex together. The possible strengthening of electrostatic interactions in vacuo will have a greater impact on complexes in which these interactions have a dominant role in stability of the complex. Studies by ESI-MS of a range of extensively characterized complexes are important to establish guidelines for the magnitude of such effects. It might be possible to measure the relative contribution of a particular interaction to stability of a complex by comparing the relative effects of various mutations on gas and solution phase dissociation constants. For example, mutation of a residue involved in an electrostatic interaction might have a greater effect on dissociation constants in the gas phase than in solution.

Materials and methods

All reagents were of analytical grade. MilliQ water was used in all experiments. Oligonucleotides (Fig. 1) were obtained from GeneWorks (Adelaide, South Australia) as the trityl on derivatives. They were deprotected using standard procedures and purified by two stages of reverse-phase high pressure liquid chromatography as described previously (Wickham et al. 1995). Concentrations of single-stranded oligonucleotides were estimated by measurement of ultraviolet light absorbance at 260 nm using values of ε_{260} for adenine, guanine, cytosine, and thymine of 15,400,

11,700, 7300, and 8300 M^{-1} cm^{-1}, respectively (Sambrook et al. 1989).

Characterization of proteins

Unmodified Tus, his_6Tus, A173T (his_6Tus in which Ala 173 was changed to Thr), and R198A (his_6Tus in which Arg 198 was changed to Ala) were expressed in *E. coli*, purified, and stored as previously described (Neylon et al. 2000). These protein samples had been characterized previously by mass spectrometry (Neylon et al. 2000), giving masses in agreement with calculated values (Table 1). Tus concentrations were determined by measurement of ultraviolet light absorbance at 280 nm, using ε_{280} = 39,700 M^{-1} cm^{-1} (Coskun-Ari et al. 1994).

Preparation of double-stranded (ds)DNA

Complementary single-stranded oligonucleotides (2.5 mM in 0.1 M ammonium acetate at pH 8.0) were heated to ≥20°C above melting temperature and allowed to cool slowly overnight. Annealed DNA was stored at 4°C before use.

Preparation of DNA–protein complexes

In first attempts to prepare a Tus–*Ter* complex, Tus and annealed *TerB* DNA were mixed in a 1 : 1 molar ratio and dialyzed together against 10 mM ammonium acetate at pH 8.0. This resulted in ESI mass spectra that showed a mixture of ions from free Tus and the complex. In addition, a small amount of precipitate was observed in dialyzed samples. Subsequently, the complex was prepared by first dialyzing Tus (1–15 μM) against 10 mM ammonium acetate at pH 8.0, at 4°C, followed by mixing it with an equimolar amount of dsDNA (typically 500 μL of protein to 1 μL of DNA in 0.1 M NH_4OAc). The mixture was left on ice for 1.5 h before injection into the mass spectrometer. In experiments in which NH_4OAc concentration was varied, a small volume of 10 M ammonium acetate at pH 8.0 was added to the mixture 1 h before mass spectrometry. In competition experiments, the two Tus protein samples were mixed in 800 mM NH_4OAc at pH 8.0 and allowed to equilibrate for 30 min, followed by addition of an equimolar amount of dsDNA, giving a final concentration of each component of the mixture of 10 μM. The mixtures were left on ice for 1.5 h before direct injection into the mass spectrometer. ESI mass spectra of samples of free proteins used in these mixtures were acquired just before and after ESI mass spectra of mixtures to ensure that there had been no drift in calibration.

Electrospray ionization mass spectrometry

ESI mass spectra were acquired using a Qtof2 mass spectrometer (Micromass, Wyntheshawe, UK) equipped with a Z-spray electrospray ionization source. This spectrometer has an m/z range of 10,000. Samples were injected directly into the source using a Harvard Model 22 syringe pump at flow rates between 5 and 10 μL min^{-1}. The best conditions for obtaining mass spectra of the DNA–protein complex were capillary, 2.5 kV; cone, 50 V; source block temperature, 40°C; desolvation temperature, 240°C; collision energy, 20 eV; aperture, 13; and transport, 6. Spectra were acquired in positive ion mode over a m/z range of 1000–7000. Typically, 25–30 scans were summed to give representative spectra. The data were calibrated against a standard CsI solution (750 μM) over the same m/z range. The ESI spectra shown in this work and those used for measurements of intensities of ions were not subjected to background subtraction but were smoothed using a 2 × 30-m/z window and Savitzky-Golay algorithm.

Electronic supplemental material

The supplemental material includes two figures. Figure 10 shows ESI mass spectra acquired under optimized instrumental conditions of the his_6Tus–*TerB* complex (10 μM) in 800, 1400, and 2000 mM NH_4OAc at pH 8.0 showing the m/z range 1500–4500. There is a decrease in the abundance of ions from the complex with increasing NH_4OAc concentration concomitant with an increase in abundance of ions from free his_6Tus and DNA. Figure 11 shows a comparison of the relative ESI-MS response factors of free Tus and a Tus–*TerB* complex. A plot of Tus_{free}/Tus_{total} and $Tus_{complex}/Tus_{total}$ determined by comparing the relative intensities of the relevant ions in the ESI mass spectrum against the relative amounts of free and complexed Tus added to the solution. In this experiment, the his_6Tus–*TerB* complex was prepared and titrated with A173T in 800 mM NH_4OAc at pH 8.0. ESI mass spectra were acquired of mixtures in which Tus_{total} was maintained at 10 μM. The following relative amounts of A173T and his_6Tus–*TerB*, respectively, were used in this experiment: 0 and 10 μM, 2 μM and 8 μM, 4 μM and 6 μM, 6 μM and 4 μM, 8 μM and 2 μM. This experiment compares the relative ESI-MS response factors of free (A173T) and complexed Tus (his_6Tus–*TerB*).

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