Microscopic DNA Flexibility Analysis

PROBING THE BASE COMPOSITION AND ION DEPENDENCE OF MINOR GROOVE COMPRESSION WITH AN ARTIFICIAL DNA BENDING AGENT*

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We have used an artificial DNA bending agent to monitor the local flexibility of the DNA helix as a function of Mg\(^{2+}\) cation concentration, sequence, and temperature. A DNA bending agent was constructed from a pair of triple helix-forming oligonucleotides connected by a flexible polymeric linker, which, when the linker is short enough, causes a bend in a minor groove region separating the two sites of triple helix formation. The unique aspect of this system is that, since the bent region is not in direct contact with the linker or the triple helix-forming oligonucleotides, the free energy reflecting the bendability of the minor helix groove can be estimated from a comparison of binding affinity between the bent and unbent triple helices. A binding competition experiment and association and dissociation kinetic assays executed at 37 °C in the presence of 25 to 65 °C, and over a Mg\(^{2+}\) concentration.

It is important to understand the flexibility or deformability of DNA in order to understand DNA folding as occurs during transcriptional activation (1) or packaging of DNA into the nucleosome (2–4). Sequence-dependent flexibility or deformability may also play a role in the recognition of DNA-binding proteins. The elastic coil model, which has served as a good model for the flexibility of duplex DNA in solution, is based on the approximation that DNA can be regarded as a stiff elastic rod. The rigidity of a rod is generally expressed as the persistence length \(P\), which is related to its Young’s modulus. DNA flexibility for bending motion of the helix axis can be represented by the bending free energy \(\Delta G_{\text{bend}}\). According to the elastic coil model (5), the free energy for DNA bending can be expressed as follows,

\[
\Delta G_{\text{bend}} = \frac{R \cdot T \cdot P \cdot (\Delta \theta)^2}{2L}
\]

where \(R\) is the gas constant, \(T\) is the absolute temperature, \(L\) is the length of the DNA under strain, \(\Delta \theta\) is the bending angle in radians, and \(P\) is the persistence length of DNA. Early studies on DNA flexibility using optical measurements (6, 7), hydrodynamic measurements (8, 9), ring closure analyses (10), and recent DNA-stretching techniques (11) have offered excellent estimations of the persistence length \(P\) of bulk DNA, which has proved to be approximately 150 base pairs (bp)\(^3\) (500 Å) for the DNA duplex.

Recently, we developed a DNA bending system (Fig. 1a), in which a tethered triple helix-forming oligonucleotide (a tethered TFO; Fig. 1b) induces a bend in a one-helical turn duplex region intervening between two sites for triple helix formation (12). The bend angle can be systematically varied with the length of the flexible covalent linker connecting the two TFOs. A tethered TFO possessing an 18-covalent bond linker (TAI18; Fig. 1b) was shown to induce a 50–70° bend, which was the largest angle in the series investigated (12). The directionality of that bend was deduced as toward the minor groove, by modeling and by phasing analysis (12). On the other hand, a tethered TFO possessing a 44-covalent bond linker (TAI44; Fig. 1b) was found, as expected, to induce little or no bending. Unlike DNA-binding proteins, which may be able to form semistable structures, the structural motif of the triple helix is relatively rigid and simple. Thus, since the distorted duplex region of the bent TAI18 complex was the only significant difference relative to the unbent TAI44 complex, a comparison of binding free energy between the two could provide a direct estimation of the free energy required for helix bending (Fig. 1a). Previously, binding competition experiments (12) have suggested that the difference in the free energy between the bent and unbent triple helix, corresponding to 0.4–0.9 kcal/mol of bending free energy, was much smaller than the value expected from the elastic coil model (~3.5 kcal/mol, from Equation 1). Based upon that apparent discrepancy, it was proposed that bendability toward the minor helix groove might be much greater than expected from a measurement of overall helix flexibility, which includes the flexibility for bending motions toward the major helix groove and toward the phosphate backbones (12). Thus, the DNA bending agent developed by us could provide information on microscopic DNA flexibility, especially with respect to bending that compresses the minor groove of a DNA duplex.

Generally, the persistence length of the DNA duplex \(P\) is thought to depend on DNA sequence (6, 7, 13, 14), counterion concentration (15), and temperature (8). In this study, we ex-

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1 The abbreviations used are: bp, base pair(s); TFO, triple helix-forming oligonucleotide; DS, DNA duplex; TS, DNA triple helix.
exploit our bending system to address three questions pertaining to DNA flexibility. The first question is whether the free energy change required for minor groove compression, as measured in our binding assay, depends on the sequence of the distorted duplex. Conformational free energy calculations (12) and experiments to investigate the flexibility of bulk DNA (6, 7, 13, 14) have suggested that the overall free energy for DNA bending is dependent on sequence, with AT-rich sequences appearing to be somewhat more flexible than a DNA duplex with a GC-rich sequence (6, 7, 13, 14). Therefore, in our assay, we have the ability to ask if the bendability of minor groove helix is proportional to that overall sequence-dependent flexibility. The second question to be asked is how the bending free energy for minor groove compression is influenced by ion concentration. An early study by Harrington (15) showed that the persistence length \( P \) decreased with increasing ionic strength. In recent years, Strauss et al. (17) have demonstrated that asymmetric phosphate neutralization by the replacement of phosphodiester bonds to methylphosphonate bonds could induce DNA bending. Interestingly, the bend induced by such charge neutralization was reversed by the addition of multivalent cations such as Mg\(^{2+}\), Ca\(^{2+}\), and spermine (17), presumably due to neutralization of the charged backbone. Therefore, we have chosen to vary Mg\(^{2+}\) ion concentration in our assay in order to monitor its effect on minor groove compression. Finally, we have investigated the effect of the temperature on the bending free energy for minor groove compression. Bending free energy can be related to the temperature by two independent terms. First, the overall bending free energy is proportional to the absolute temperature (Equation 1). Second, measurement of transatlantal coefficients via sedimentation has suggested that the persistence length of duplex DNA decreases with increasing temperature (8), as would generally be expected from temperature-dependent reduction of Young’s modulus. In that context, we have sought to determine if local minor groove compression displays a temperature coefficient that is similar to that determined for the overall flexibility of the duplex.

EXPERIMENTAL PROCEDURES AND THEORY

Materials—Syntheses of oligonucleotides and the construction of the plasmids (pBend-NOBAT, NOBMIX, and NOBGC) were carried out as described previously (12). T, polynucleotide kinase and DNA polymerase I Klenow fragment were purchased from Life Technologies, Inc. Restriction enzymes and calf intestine alkaline phosphatase were from Boehringer Mannheim. [\(^{32}\)P]-ATP and DNA polymerase I Klenow fragment were purchased from Du Pont.

Equilibrium Binding Analyses—A restriction fragment excised from the pBend vector by treatment with EcoRV (pBend4-NOBAT, NOBMIX, or NOBGC) was dephosphorylated by calf intestine alkaline phosphatase and was end-labeled with [\(^{32}\)P]ATP and T, polynucleotide kinase (18). This labeled DNA (~1 nm) was incubated with TFO in the standard binding buffer (100 \( \mu \)l of 10 mm Tris-HCl (pH 7.6) and 10 mm MgCl\(_2\)) for approximately 20 h. At high incubation temperatures, mineral oil (2–3 \( \mu \)l) was overlaid to prevent evaporation. The resulting triple helix solution was loaded onto a polyacrylamide gel (29.1% cross-linking), and the gel was run (12.5 V/cm) in 89 mm Tris borate buffer containing 10 mm MgCl\(_2\) (TBM) for 4 h. The dried gel was visualized by autoradiography and was quantified with a Betascope 603 blot analyzer. Curve fitting to the data was executed by using Deltagraph Prodl software on a Macintosh Ici, in which nonlinear least square analysis was used. The equilibrium dissociation constant was calculated as follows. If the approximation of the all or none transition of the triple helix is correct, the binding isotherm of the triple helix formation can be expressed as follows,

\[
\text{DS} + \text{TFO} \rightleftharpoons \text{TS}
\]

\[
K_1 = \frac{[\text{DS}][\text{TFO}]}{[\text{TS}]}
\]  

(Eq. 2) where DS and TS represent the DNA duplex and the triple helix, respectively. Brackets represent the concentration of each fraction. The equilibrium dissociation constant \( K_1 \) is expressed by Equation 2, where \( k_1 \) is the association rate constant and \( k_{-1} \) is the dissociation rate constant. In the case where the TFO is in excess over DNA duplex bearing the TFO binding site and when duplex concentration [DS]\(_0\) is lower than the \( K_d \), the fraction of double helix bound as triple helix can be expressed as a function of the concentration of the added TFO, [TFO]\(_0\),

\[
\frac{[\text{TS}]}{[\text{DS}]} = \frac{a[T\text{FO}]_0}{K_1 + [\text{TFO}]_0}
\]  

(provided that \( a \leq 1 \) ) (Eq. 3)

where [TS]/[DS]\(_{total}\) is the measured distribution of bound versus total duplex, and \( a \) is the experimental correction factor; ideally \( a \) should be 1. The values of \( a \) were obtained from 0.93 to 1.00, suggesting sufficient experimental accuracy of the titration experiments. Competition assays between the bent and unbent triple helix formations were carried out as described previously (12) and were analyzed as follows. When the competition reaction reaches equilibrium, the binding isotherm is as follows,

\[
\frac{[\text{TS}]}{[\text{DS}]} = \frac{[\text{TFO}_\text{bend}]}{[\text{TFO}_\text{unbent}]}
\]

where \( K_d \) (bent triple) and \( K_d \) (unbent triple) are the equilibrium dissociation constants of the bent triple helix and unbent triple helix, respectively. Simple division of \( K_d \) (bent triple) by \( K_d \) (unbent triple) gives the following,

\[
\frac{[\text{DS}][\text{TFO}_\text{bend}][\text{TFO}_\text{unbent}]}{[\text{TFO}_\text{bend}][\text{TFO}_\text{unbent}]} = \frac{[\text{TS}]}{[\text{DS}]}
\]

(Eq. 4) At the midpoint of the competition, [TS]\(_{unbent}\) = [TS]\(_{bend}\),

\[
\frac{[\text{DS}][\text{TFO}_\text{bend}][\text{TFO}_\text{unbent}]}{[\text{TFO}_\text{bend}][\text{TFO}_\text{unbent}]} = \frac{[\text{TS}]}{[\text{DS}]}
\]

(Eq. 5) As the free energy change in the triple helix formation is given by the following,

\[
\Delta G_{\text{triplex}} = RT \ln K_d(\text{triplex})
\]

(Eq. 6) the bending free energy \( \Delta G_{\text{bend}} \) should be as follows.

\[
\Delta G_{\text{bend}} = \Delta G_{\text{triplex}} - \Delta G_{\text{unbent}} = RT \ln \frac{K_d(\text{bent triple})}{K_d(\text{unbent triple})}
\]

(Eq. 7) At the midpoint of the competition, substituting by Equation 7,

\[
\Delta G_{\text{bend}} = RT \ln \frac{[\text{TFO}_\text{bend}]}{[\text{TFO}_\text{unbent}]}
\]

(Eq. 8) By such an analysis, the bending free energy \( \Delta G_{\text{bend}} \) can be evaluated directly from the ratio of TFO concentrations at the midpoint. Association Kinetics—A synthetic DNA duplex generated by the hybridization of two complementary oligonucleotides (48-mers) was labeled with [\(^{32}\)P]dTATP and DNA polymerase I Klenow fragment under standard conditions (18). The labeled 50 bp duplex DNA (~1 nm) was incubated with TFO in the standard binding buffer at 37 °C for each incubation time, and loaded onto 12% non-denaturating polyacrylamide gel electrophoresis containing TBM. The gel was run in TBM buffer (11 V/cm) for 2–3 h. The dried gel was visualized by autoradiography and was quantified with the Betascope. Data were analyzed according to a simple first order kinetic model. If the concentration of TFO [TFO]\(_0\) added to the reaction is in excess (>10-fold) over the concentration of duplex [DS]\(_0\), the change of the concentration of TFO [TFO]\(_0\) can be neglected during the time course, and TFO binding kinetics can be treated in the context of a pseudo-first-order reaction.

\[
\ln \frac{[\text{DS}]}{[\text{DS}]} = -k_\text{obs} \cdot t
\]

(Eq. 11) where \( k_\text{obs} = k_1 \cdot [\text{TFO}]_0 + k_{-1} \) (Eq. 12)
where \([\text{DS}]_{eq}\) is the fraction of the duplex remaining unbound at equilibrium, which can be calculated from the measured equilibrium constant, \([\text{DS}]_0\) is the fraction of the duplex at time zero, and \([\text{DS}]\) is the fraction of duplex at time \(t\). \(k_{\text{obs}}\) is the observed pseudo-first-order association kinetic constant. The slope of the plot of the following,

\[
\ln \left( \frac{\text{[TS]} - \text{[TS]}_0}{\text{[DS]} - \text{[DS]}_0} \right)
\]

against time \((t)\) should give a \(k_{\text{obs}}\) at each \([\text{TFO}]_0\), and a \(k_1\) can be obtained from the plot of \(k_{\text{obs}}\) versus \([\text{TFO}]_0\).

**Dissociation Kinetics**—The labeled synthetic duplex DNA (50 bp) was incubated with TFO (100 nM) at 37 °C for approximately 20 h in the standard binding buffer. The dissociation reaction was initiated by the addition of two 15-mers, designed as a Watson-Crick complement of the TFO (2 μM each). The resulting solution was analyzed by electrophoresis as it was for the association kinetics. Assuming that the dissociation reaction of the triple helix is irreversible and the oligomer complements are in large excess, the dissociation reaction can be modeled as first order, and the dissociation process can be expressed as follows.

\[
\ln \left( \frac{\text{[TS]} - \text{[TS]}_0}{\text{[DS]} - \text{[DS]}_0} \right)
\]

The plot of \(\ln([\text{TS}]/[\text{TS}]_0)\) versus time \((t)\) should yield the first order dissociation rate constant \(k_{-1}\).

**Relation between Equilibrium Dissociation Constant and Concentration of \(\text{Mg}^{2+}\)**—If the effect of \(\text{Mg}^{2+}\) binding on the transition between the triple helix and duplex is explained by ion condensation theory and mass action, the change of measured equilibrium dissociation constant as a function of added \(\text{Mg}^{2+}\) should be expressed as follows,

\[
\Delta n = \frac{\partial \ln K_d}{\partial \ln [\text{Mg}^{2+}]} \times \Delta [\text{Mg}^{2+}]/2
\]

Where \(\Delta n\) is the net change in bound ion equivalents, resulting from binding of the oligonucleotide.

**RESULTS**

**Determination of Apparent Dissociation Constants by Gel Mobility Shift Experiments**—Titration experiments using the gel mobility shift assay (19, 20) were executed to evaluate the binding affinity of tethered TFOs (Fig. 1b) for a 171-bp duplex DNA fragment. Fig. 2a shows a typical mobility shift analysis in polyacrylamide gel electrophoresis. As the concentration of TFO (TAI18 and TAI44, Fig. 1b) increases, the uncomplexed 171-bp duplex (NOBMIX, Fig. 1c) is converted to a band with slower mobility. By reference to previous studies of the triple helix formation (19, 20), the retarded band should correspond to the triple helix. In principle, there are four possible complexes in this binding assay in which TFO is in excess over the duplex (~1 nM). The first alternative is the perfectly bound 1:1 complex of a tethered TFO and the binding site, which is expected from the simple modeling (Fig. 1a). The second and third possible complexes are partially bound 1:1 complexes, in which either of two triple helix regions is dissociated. The last alternative is a 2:1 complex, in which two molecules of the tethered TFO are bound to one binding site.

We conclude that the observed complex is the perfectly bound 1:1 complex for two reasons. First, the electrophoretic mobility of the alternative complexes should be nearly independent of a change in the linker length in the tethered TFO, since the linker is very small as compared with a 171-bp duplex. Even if the linker were to influence the electrophoretic mobility, the sense of the effect should have been a modest decrease in electrophoretic mobility with increasing size of the linker span. This is inconsistent with experimental data, as described previously (12); i.e. the TAI18 (18 bonds) complex has significantly slower mobility than the TAI44 complex (Fig. 2a). That behavior is consistent with the fact that the bent DNA has anomalously slow mobility in polyacrylamide gel electrophoresis (21, 22). Secondarily, binding stoichiometry analysis of TAI18 and TAI44 (500 nM of each strand) has shown that, at saturation, the predominant structure of the resulting triple helix is a discrete 1:1 complex and a trace amount of a more significantly retarded complex, perhaps a 2:2 complex (data not shown). It should be noted that the partially dissociated 1:1 complex, which is a possible structure in the process of forming perfectly bound 1:1 complex, is not observed during equilibrium analysis (Fig. 2a). This finding suggests that, for these linked TFOs, binding may be considered to occur as a bimolecular association process.

Fig. 2b is a plot of \([\text{TS}]/[\text{DS}]_{\text{total}}\) versus \([\text{TFO}]_0\) as obtained by
the gel shift method for TAI18 and TAI44. Fitting of the data to Equation 3, which is based on the approximation of the all or none transition of the triple helix (see “Experimental Procedures and Theory”), gives the curve in Fig. 2b. Analysis of this kind has been performed for the entire series of linked TFOs from 18 to 44 rotatable bonds in the linker span. Previous bending structure analyses had shown that this series of ligands induced progressively larger bending of the duplex (12). Measured $K_d$ values determined as in Fig. 2b along with estimates of the experimentally determined bending angle are presented in Table I. The $K_d$ of each TFO exhibited in Table I is associated with approximately $-10$ kcal/mol as the free energy change of triple helix formation. It is interesting to note that detailed analysis of the data in Table I reveals that the relation between binding affinity and linker span appears to be biphasic, with an indication of a shallow local maximum near 24 bonds of linker span. Simple elastic theory would have predicted a continuous, more or less quadratic dependence saturating at length greater than 25 bonds. The origin of the small increase in binding affinity that occurs in the range from 25 to 44 bonds cannot be ascertained from the data at hand, but it could be due to second order change or configurational effects. Independent of such second-order concerns, it should be noted that the difference in the $K_d$ among the triple helices is considerably smaller than that expected from a simple elastic coil model (Table I, compare the second and the bottom rows). These results are qualitatively consistent with results obtained from a binding competition analysis previously described, which showed that the difference in the binding affinity between the bent and unbent complex was very small (12).

**Sequence Dependence of Minor Helix Groove Compression**—As described above, the unbent triple helix should be more stable than the bent triple helix, since the strain energy required to accommodate bending should be deducted from the free energy of binding. To a very good approximation, the binding free energy difference between the bent and unbent triple helix should be equivalent to the free energy of bending distortion. Circular permutation analysis previously executed by us has suggested that the bending induced by the tethered TFO is located in the intervening duplex region (12). In order to elucidate the dependence of bending free energy on the sequence, binding competition experiments were performed on three kinds of 171-bp DNA fragment, which differ in the AT content of the sequence in the 10-bp spacer region that separates the two sites of triple helix formation (Fig. 1c). The reduced mobility of the bent complex with TAI18 was exploited to detect concurrently both the bent and unbent complex on the same polyacrylamide gel. As seen in Fig. 3a (first column), the unbent TAI44 complex (100 nM of TAI44) displays faster electrophoretic mobility (arrow A). Upon addition of increasing amounts of TAI18 in the 10 nM to 1 nM range, the unbent complex is converted to the bent TAI18 complex with slower mobility (arrow A), with a midpoint of approximately 200 nM of

![Fig. 2. Dissociation constants via gel shift analysis.](image)

**TABLE I**

| Binding constant | TAI18 | TAI22 | TAI24 | TAI30 | TAI33 | TAI44 |
|------------------|-------|-------|-------|-------|-------|-------|
| Observed $K_d$ (nM)$^a$ | $20 \pm 3$ | $27 \pm 3$ | $57 \pm 12$ | $30 \pm 3$ | $16 \pm 3$ | $15 \pm 3$ |
| $K_{E_TFO}/K_{E_TAI44}$$^b$ | 1.3 | 1.8 | 3.8 | 2.0 | 1.1 | 1 |
| Bending angle (degrees)$^c$ | 53 | 48 | 39 | 27 | 22 | 0 |
| Calculated $\Delta G_{bending}$ (kcal/mol)$^d$ | 4.1 | 3.4 | 2.2 | 1.1 | 0.7 | 0 |
| Expected $K_{E_TFO}/K_{E_TAI44}$ | 800 | 250 | 36 | 6.0 | 3.1 | 1 |

$^a$ Observed equilibrium dissociation constants ($K_d$) for TFO binding were obtained from titration experiments of the 171-bp EcoRV fragment of pBend4-NOBMIX (50% spacer AT content) with tethered TFOs with varying linker span as in Fig. 2.

$^b$ The ratio of the $K_d$ for triple helix formation in NOBMIX relative to that for TAI44.

$^c$ Bending angles are those measured previously for these complexes (12).

$^d$ Calculated bending free energy $\Delta G$ was obtained from elastic coil theory Equation 1, assuming a persistence length of 150 bp for the 10-bp spacer region and the above set of measured bending angles.

The expected $K_{E_TFO}/K_{E_TAI44}$ ratio refers to the ratio of measured dissociation constant as in Footnote $b$, relative to that for the TAI44 complex, which would be expected if the measured data were a simple function of bulk flexibility parameters.
added TAI18. Surprisingly, almost no difference is seen in the concentration of TAI18 required to displace TAI44 among AT- and GC-rich spacers (Table II). In the context of Equation 10, the data obtained from the competition experiments in Fig. 3 give 0.4–0.7 kcal/mol as the free energy required for the 50–70° minor groove bending of all three sequences at 37 °C. These values are consistent with the direct titration data of Table I. In order to confirm the surprisingly high flexibility obtained from direct and competition analyses, we have chosen to augment equilibrium measurements by monitoring the kinetics of the TFO-induced bending process.

Association Kinetics of Bent and Unbent Triple Helix Formation—Generally, measurement of association and dissociation kinetics can be used to confirm a reaction mechanism and provide an estimate of binding affinity, which can complement that of a direct equilibrium measurement. In order to confirm the unexpectedly small difference in the equilibrium binding constant between the unbent and bent triple helix complexes, kinetic analysis was carried out at 37 °C. NOBAT (AT-rich sequence, Fig. 2c) and NOBGC (GC-rich sequence, Fig. 2c) were chosen as binding sites, since they were expected to be the most flexible and most rigid sequence, respectively, based upon previous reports (6, 7, 13, 14). A short synthetic duplex (50 bp) bearing each binding site was used for analysis. The slow kinetics of triple helix formation allowed us to utilize gel electrophoresis to measure the kinetics of the triple helix formation. To the best of our knowledge, the detailed mechanism of simple triple helix formation remains unclear. However, assuming that triple helix formation can be regarded as a one-step reaction of a duplex and a TFO (Fig. 1a), the process of the triple helix formation can be treated as a second-order reaction. It should be emphasized that this approximation may not reflect the real process of the triple helix formation. However, a similar approximation has provided an estimation of kinetic parameters of purine-pyrimidine-pyrimidine type parallel triple helix formation (23, 24). Analogous to our current interest in oligonucleotide chimeras, the hybridization of a similar tethered oligonucleotide to noncontiguous sites on single stranded RNA has previously been successfully treated as a one-step reaction, and the kinetic constants for the heteroduplex formation were estimated (25). Fig. 4a shows typical kinetic behavior of this TFO association reaction as monitored by polyacrylamide gel electrophoresis. The retarded band corresponding to the triple helix increases over the 25-min incubation time. At time 0, immediately after the addition of TFO, the triple helix band was not detected. This observation, which was general in all executed experiments of association kinetics, shows that, in this time scale and over the range of TFO concentration tested, triple helix formation is slow enough to be measured by the gel shift method. In addition, the partially dissociated 1:1 complex, which could be an intermediate in the process of perfectly bound 1:1 complex formation, was not observed. Data as in Fig. 4a have been quantified as required by Equation 11 and have been plotted in a semilogarithmic fashion in Fig. 4b and c. The slope of such plots, which are a direct measure of $k_{\text{obs}}$, have been plotted versus added TFO concentration in Fig. 4d. From the plot of $k_{\text{obs}}$ versus the [TFO]₀ (Fig. 4d), the second-order association constant ($k_{\text{a}}$) of triple helix formation was obtained, and it is catalogued in Table II. As seen in Fig. 4a, b and c, and Table II, the rate of bent triple helix formation (TAI18 complex) is slower than that of the unbent triple helix (TAI44-complex) by about a factor of 2. The data are consistent with the expectation that, in forming the bent triple helix, TAI18 might “wait” for the bending motion of the substrate duplex. However, the difference in measured rate for bent versus unbent complex is small, which is again consistent with the small measured differences in equilibrium constant. Interestingly, the pair of unbent (TAI44) complexes (Table II) display measured association constants that are identical to better than experimental accuracy for binding sites with AT- and GC-rich spacers (Table II). This serves as an internal control for the accuracy of these measurements, since the two complexes differ only in the (un-
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Fig. 4. Association kinetics. a, autoradiography of the association experiments. Top, the association reaction of TAI18 to a 50-bp duplex. Bottom, the association reaction of TAI44. TFO (50 nM) was incubated with 3'-end-labeled synthetic duplex (1 nM) at 37 °C for 0, 5, 10, 15, 20, and 25 min as shown at the top. The TS arrow indicates the triple helix band, and the DS arrow indicates that of the unbound 50-bp duplex. b and c, determination of observed association kinetic constants (b for TAI18 and c for TAI44). The natural logarithm of \( \frac{[DS]_{eq} - [DS]_0}{[DS]_{eq} - [DS]_u} \) is plotted against the incubation time (min). The concentration of added TFO is 20 nM (closed squares), 30 nM (open circles), 50 nM (closed triangles), 80 nM (closed diamonds), 100 nM (open squares), 120 nM (open circles), 150 nM (open triangles), and 200 nM (closed diamonds). The lines show the fitting of data points to Equation 11. d, determination of association kinetic constants (\( k_{ob} \)). The observed association constants (\( k_{ob} \)) obtained from Fig. 4, b and c, and Equation 11 are plotted against the concentration of the added TFO. Cicles, the plot of \( k_{ob} \), for the binding of TAI18 to NOBGC (the duplex with 100% GC content in the intervening region); squares, the plot of \( k_{ob} \), for the binding of TAI44 to NOBGC. The error bars refer to the S.E. derived from the linear regression in Fig. 4, b and c, according to the equation.

\[
\text{S.E.} = 100 \cdot \frac{1}{n} \sum (\theta_p - \theta_{\text{fit}}) \quad \text{(Eq. 17)}
\]

where \( \theta_p \) is the triple helix fraction determined from experiments, \( \theta_{\text{fit}} \) is that value derived from the line fit, and \( n \) is the number of data points.
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**Table II**

Summary of kinetic and equilibrium parameters as a function of base composition

Kinetic parameters were obtained by analysis of data obtained at 37 °C, as in Figs. 4 and 5. Calculated equilibrium dissociation constants are obtained by dividing experimental rate constant \( k_a \) by \( k_{-a} \). Measured equilibrium dissociation constants were obtained at 37 °C from gel titration analysis as in Fig. 2. Bending free energy \( \Delta G_{\text{bend}} \) is calculated by using Equation 9 and the ratio of calculated equilibrium constants \( (K_{b}^{\text{TAI18}} / K_{b}^{\text{TAI44}}) \).

| DNA Oligonucleotide | Association rate constant \( (k_a) \) \( \text{M}^{-1} \cdot \text{s}^{-1} \) | Dissociation rate constant \( (k_{-a}) \) \( \text{s}^{-1} \) | Calculated equilibrium dissociation constant \( (K_{b}) \) \( \text{nM} \) | Measured equilibrium dissociation constant \( (K_{b}) \) \( \text{nM} \) | \( K_{b}^{\text{TAI18}} / K_{b}^{\text{TAI44}} \) | Bending free energy \( (\Delta G_{\text{bend}}) \) kcal/mol |
|---------------------|-----------------------------------|---------------------|---------------------|---------------------|---------------------|---------------------|
| NOBAT TAI18         | \( (4.6 \pm 0.4) \times 10^7 \)   | \( (8.1 \pm 0.8) \times 10^{-5} \) | 18                  | 12                  | 5.1                 | 1.0                               |
| NOBAT TAI44         | \( (7.4 \pm 0.6) \times 10^7 \)   | \( (2.6 \pm 0.6) \times 10^{-5} \) | 3.5                 | 6.1                 | (2.0)\( ^a \)        | (0.4)\( ^a \)           |
| NOBG C TAI18        | \( (3.2 \pm 0.3) \times 10^7 \)   | \( (6.9 \pm 1.5) \times 10^{-5} \) | 22                  | 14                  | 4.5                 | 0.9                               |
| NOBG C TAI44        | \( (7.2 \pm 0.9) \times 10^7 \)   | \( (3.5 \pm 0.8) \times 10^{-5} \) | 4.9                 | 6.8                 | (2.1)\( ^a \)        | (0.5)\( ^a \)           |

Note: The values in parentheses were calculated using the corresponding measured equilibrium dissociation constant ratio.

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**Fig. 5.** Dissociation Kinetics. *a*, autoradiography of the dissociation reaction. *Top*, the dissociation reaction of the triple helix formed by TAI18 and the 50-mer duplex (NOBAT, 100% AT content in the spacer duplex). *Bottom*, the dissociation reaction of TAI44-NOBAT triple helix.

*Fig. 6.** Effect of Mg\(^{2+}\) concentration on the stability of the bent and unbent complexes. *a*, titration curves for the bent and unbent triple helix formation as a function of Mg\(^{2+}\) ion concentration. Titration was performed with the 171-bp duplex fragment (NOBMIX, 50% AT content) with TAI18 (open symbols) and TAI44 (closed symbols) in the presence of 0.3 mM (diamonds), 1 mM (triangles), 3 mM (circles), or 10 mM (squares) of MgCl\(_2\) plus 10 mM Tris-HCl, pH 7.6. Error bars show the standard deviation obtained from three different experiments. Data have been fit using Equation 3. *b*, plot of the logarithm of the apparent equilibrium dissociation constant obtained from curve fits in Fig. 6a versus the logarithm of the Mg\(^{2+}\) concentration in the triple helix solution. Open circles are the plot for TAI18 complex; closed squares are the plot for the TAI44 complex. The lines represent the best linear squares fit of the data to Equation 15.

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The values in parentheses were calculated using the corresponding measured equilibrium dissociation constant ratio.
nearly constant. This suggests that the apparent similarity in binding stability of bent and unbent complexes, which was observed at 37 °C, appears to manifest over a wide temperature range. This observation was confirmed by the competition experiments carried out over the same temperature range (Fig. 7c). The temperature coefficient of the binding reaction for both bent and unbent complexes is found to give rise to increasing binding affinity at elevated temperature. This unusual temperature effect has been previously described for purine-purine-pyrimidine triple helices and has been ascribed to intermolecular structure formation by the unbound TFO (27) with the intermolecular folding being more temperature-dependent than the equilibrium leading to triple helix formation. In spite of that complexity, it should be noted that the small difference in $K_d$ values for the bent and unbent complexes are observed to remain constant over the entire temperature range from 25 to 67 °C (Fig. 7b).

DISCUSSION

Previously (12), based upon a comparison of bending structure analysis and binding competition measurements, we have concluded that for a random sequence DNA segment, compression of the minor helix groove appears to involve energies that are 5–10-fold smaller than previously assumed, based upon extrapolation of bulk flexibility parameters.

The apparently small difference in binding free energy between bent and unbent complexes is so surprising that alternative explanations should be evaluated. One possible explanation is the formal possibility that, upon forming the triple helix, the region immediately adjacent to the binding site might somehow assume a distorted, highly flexible structure. In order to address this possibility, we have employed ring closure analysis of the bent complexes, which has been shown to be a sensitive measure of localized flexibility change (28). Ring closure analysis using a 171-bp duplex was carried out by us (data not shown) and has shown that binding of the unlinked TFOs or the TAI44 complex appeared to induce a modest stiffening of the duplex, rather than an increase in DNA flexibility. This observation will be presented elsewhere, as part of a more thorough analysis of binding by these complexes. However, for now, it should be noted that the observation of TFO-induced stiffening is consistent with the results of Maher et al. (24), who have concluded, based upon circular permutation analysis, that pyrimidine-purine-pyrimidine triple helices produce a significant net stiffening of the duplex. Based upon these published and unpublished observations of the general finding that triple helix formation greatly stabilized the duplex relative to the helix strand transition, at present, the possibility of triple helix-induced DNA flexibility increase seems unlikely.

In the current study, we have extended the initial work by comparing equilibrium binding parameters (Figs. 2 and 3) and kinetic parameters (Figs. 4 and 5) for TFO-induced bending of both AT-rich and GC-rich helix elements. These additional data confirm the previous conclusion that induction of a 50–70° bend via tethered TFO binding is associated with an unexpectedly small distortional energy.

Given the small apparent energy of minor groove compression, it is perhaps not surprising that we have been able to detect only a small base composition dependence. Superficially, this very small base sequence composition dependence appears of TAI18. The concentration of TAI18 is 5 µM for the experiment at 25 °C and 10 nM for the experiments at 37 °C, 42 °C, and 68 °C. The plots are the average of two independent experiments at 25, 42, and 68 °C and three independent experiments at 37 °C.
to contradict published data. Calculations by Sarai et al. (16) have suggested that the work required to bend DNA may depend on bending direction, with AT-rich DNA favoring minor groove compression over major groove compression, the opposite holding true for GC-rich DNA. This calculated anisotropy is nicely consistent with the sequence-dependent "bendability" observed by Crothers et al. (29) in analysis of CAP protein-DNA interaction. Similarly, Zhurkin et al. (30, 31) have shown that the intrinsic bend detected in contiguous oligo(dA)oligo(dT) tracts can be explained by a directional preference for fluctuation of minor groove versus major groove compression. Those published measurements and calculations are focused on the difference between minor groove and major groove compression rather than the absolute work required to obtain bending deformation. As such, these data are not directly applicable to the results of our analysis of bending.

A somewhat clearer correlation with our work can be obtained by inspection of the binding 434 repressor to its cognate operator sites (32–35). X-ray crystallography has revealed that, upon binding repressor, the center of the operator becomes overtwisted and bent so as to compress the minor helix groove at sites that are not in direct contact with protein (32). Koudelka and Ptashne (33, 34) have observed that conversion of the central region from an AT-rich to a GC-rich sequence diminished binding affinity for the protein by about 50-fold. Based upon binding analyses of singly nicked operator sites (34) and the linking number of circularized DNA by the ligase reaction, Koudelka and Ptashne have suggested that the effect of base substitution within the central operator region is dominated by the base composition dependence of DNA twist deformation, whereas, by comparison, they concluded that the base composition dependence of minor groove compression appeared to be small for the 434 repressor-operator interaction (35).

The agents that we have discussed were designed to bend but not twist the duplex (12). Detailed analysis of binding to a phase-sensitive vector (12)2 confirms that measurable twist deformation is not produced. Therefore, our data cannot yet provide confirmation of the base composition dependence of DNA twist deformation, which was proposed by Koudelka et al. (34, 35). However, our studies do suggest that minor groove compression of the magnitude seen in the 434 repressor-operator complex should have been associated with an unmeasurably small deformation energy. As such our work serves to confirm, in part, those previous conclusions (34, 35).

Finally, optical and hydrodynamic studies have provided evidence that the overall bending flexibility of duplex DNA appears to be base composition-dependent, with GC-rich DNA being stiffer than seen for AT-rich duplexes (13). In order to be consistent with the conclusions drawn from our work and that of Ptashne and Koudelka (34, 35), it is necessary to conclude that the observed base sequence dependence of overall DNA bending flexibility in solution results from degrees of freedom other than minor groove compression, possibly the base composition dependence of major groove compression. Additional work is in progress to confirm that idea.

Titration experiments with the tethered TFOs at different Mg2+ concentration (Fig. 6) suggest that the bendability of the minor helix groove is not influenced by the Mg2+ ion over the range from 0.3 to 10 mM. Unfortunately, alkali metal ions such as Na+ or K+ could not be used instead of Mg2+ in these studies, because Mg2+ ion is essential for the stable formation of purine-purine-pyrimidine type triple helices (19, 20), and alkali metal ions, especially K+, are known to induce guanine-thymine-rich oligonucleotides to form tetraplex structures, intramolecular and intermolecular, which would have additionally complicated data interpretation (36–38). With these caveats in mind, it remains reasonable to conclude that a relatively high concentration of Mg2+ cation does not contribute to the unusual minor groove flexibility we have observed.

The very small temperature dependence for minor groove bending flexibility that we have detected (Fig. 7b) is consistent with the results from hydrodynamic estimates of the persistence length of bulk DNA. In that study, the data showed that the persistence length changed by only about 10% from 5 to 49°C (8). The very small change expected from these studies is too small to have produced a meaningful difference in our system. In fact, the competition assay in Fig. 7c has established that the difference in the bending affinity between the bent and unbent complex is virtually independent of the temperature between 25 and 65°C (Fig. 7b). It should be noted that the temperature dependence data can be used to rule out the possibility that the slow kinetics of the triple helix formation somehow compromised our affinity measurements, because a fast equilibrium between two states should have been achieved more easily at a higher temperature.

One striking finding in the study of the temperature dependence of tethered TFO binding (Fig. 7) is that the apparent stability of the triple helix has increased with raising temperature. If data are applied to the van’t Hoff relation, the apparent change of the enthalpy (ΔH°) during triple helix formation can be expressed as follows,

$$\frac{\Delta H^0}{\Delta H} = \frac{\Delta H^0}{R}$$

(Eq. 16)

where $K_a$ is the apparent association constant of triple helix formation. From the plot of ln $K_a$ versus 1/T (Fig. 7b), the apparent enthalpy change (ΔH°) is estimated to be positive for both TAI18 binding (34 ± 15 kcal/mol) and TAI44 binding (38 ± 10 kcal/mol). Here, it is assumed that the enthalpy does not change over this range of temperature (298–319 K). Independently, Pilch et al. (39) obtained the thermodynamic parameters of purine-purine-pyrimidine type triple helix from thermal melting profiles (39). In that study, the transition from the triple helix to the duplex was not observed; however, the enthalpy change from triple helix structure to three single strands was estimated. In that study, simple subtraction of ΔH for the dissociation of the triple helix to the three single strands from ΔH for the Watson-Crick duplex dissociation allowed the estimation of the enthalpy of forming of the triple helix from duplex and TFO, which was found to give a negative value. The large difference between the apparent ΔH measured in our studies and that deduced from thermal denaturation is reminiscent of differences that have been previously detected in the study of double helix formation. Vesnäver et al. (40) have detected similar discrepancies when comparing the enthalpy of duplex formation from differential scanning calorimetry, versus the enthalpy obtained at lower temperature via isothermal batch calorimetry. They have proposed that the measured difference results from structural order within the individual single strands at low temperature, which contributes much less to enthalpy values when they are monitored near to the melting temperature (Tm). Similarly, based on the temperature dependence of binding data at low temperature, Noonberg and colleagues (27) have observed a positive temperature coefficient for simple triple helix formation. As for duplex formation, they have ascribed this positive temperature coefficient to the contribution, at low temperature, of secondary structure within the interacting strands (27). Based upon the formal similarity of our findings with those obtained for duplex (40) and triplex

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2T. Akiyama and M. E. Hogan, unpublished results.
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formation (27), we tentatively ascribe the unusual temperature dependence of tethered TFO formation to that sort of secondary coupling effect.

CONCLUSION

A body of evidence described here suggests that compression of the minor helix groove is associated with much greater flexibility than previously expected. This unexpected flexibility persists over a wide range of the temperature and salt concentration and is practically independent of the sequence. However, we should emphasize that this system can only reveal the flexibility with respect to twisting motion or concerning the work required to compress the major groove. When our conclusions are paired with those of others (34, 35), a model emerges, which suggests that DNA flexibility may be more anisotropic than previously believed, with special emphasis placed on the ease with which the minor groove can be compressed, as a result of random thermal perturbation or the binding of ligands such as a regulatory protein.

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