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Modified nucleoside-dependent transition metal binding to DNA analogs of the tRNA anticodon stem/loop domain

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Biologically active DNA analogs of $tRNA^{Phe}$ ($tDNA^{Phe}$) were used to investigate metal ion interaction with tRNA-like structures lacking the 2'OH. Binding of Mg^{2+} to the 76 oligonucleotide $tDNA^{Phe}$, monitored by circular dichroism spectroscopy, increased base stacking and thus the conformational stability of the molecule. Mg^{2+} binding was dependent on a $d(m^5C)$ in the anticodon region. In contrast to Mg^{2+}, Cd^{2+} decreased base stacking interactions, thereby destabilizing the molecule. Since alterations in the anticodon region contributed to most of the spectral changes observed, detailed studies were conducted with anticodon hairpin heptadecamers ($tDNA^{Phe}_{AC}$). The conformation of $tDNA^{Phe}_{AC}$–$d(m^5C)$ in the presence of 1 mM Cd^{2+}, Co^{2+}, Cr^{2+}, Cu^{2+}, Ni^{2+}, Pb^{2+}, VO^{2+} or Zn^{2+} differed significantly from that of the biologically active structure resulting from interaction with Mg^{2+}, Mn^{2+} or Ca^{2+}. Nanomolar concentrations of the transition metals were sufficient to denature the $tDNA^{Phe}_{AC}$–$d(m^5C)$ structure without catalyzing cleavage of the oligonucleotide. In the absence of Mg^{2+} and at $[Cd^{2+}]$ to $[tDNA^{Phe}_{AC}$–$d(m^5C)]$ ratios of approximately 0.2–1.0, $tDNA^{Phe}_{AC}$–$d(m^5C_{40})$ formed a stable conformation with one Cd^{2+} bound with a $K_d = 3.7 \times 10^{-7}$ M. In contrast to Mg^{2+}, Cd^{2+} altered the DNA analogs without discriminating between modified and unmodified $tDNA^{Phe}_{AC}$. This ability of transition metals to disrupt higher order DNA structures, and possibly RNA, at μM concentrations, *in vitro*, demonstrates that these structures are potential targets in chronic metal exposure, *in vivo*.

Keywords: tRNA, tDNA, anticodon domain, transition metal ion effects, spectroscopic characterization

Introduction

Post-transcriptional modification is important for facilitating metal ion binding to rRNAs. Both structure (Chen *et al.* 1993, Yue *et al.* 1994) and function (Sampson & Uhlenbeck 1988) of unmodified transcripts of tRNAs require concentrations of Mg^{2+} that are 10- to 50-fold higher than that of cytoplasmic Mg^{2+}. Magnesium, at 1 mM (Murphy *et al.* 1989), is the most prevalent intracellular divalent metal ion and has a high affinity for polynucleotides (Cowan *et al.* 1993). Native, fully modified yeast $tRNA^{Phe}$ has four tight Mg^{2+} binding sites (Quigley *et al.* 1978), and has been shown to bind other ions such as Mn^{2+} (Chao & Kearns 1977), Eu^{3+}, Dy^{3+} and Pr^{3+} (Jones & Kearns 1974). However, the degree to which the ribose 2'OH and modified nucleosides are important to metal ion coordination by RNA is not understood. Biologically active DNA analogs of RNA can be used to investigate the importance of both the 2'OH and modifications to metal ion binding. Substitutions of deoxynucleotides for ribonucleotides in stems of the hammerhead ribozyme, the enzymatic activity of which is dependent on metal ions, have been analyzed for enhanced catalytic efficiency and increased stability (Taylor *et al.* 1992). In the presence of various divalent metal ions, DNA substitutions in non-catalytic-loop regions of the hammerhead ribozyme increased ribozyme activity (Sawata *et al.* 1993). When exposed to organometallic reagents that cleave the nucleic acid backbone, the DNA analogs of tRNA yield fragmentation patterns similar to that of the native tRNA indicating structural similarities (Lim & Barton 1993). Results of enzymatic cleavages of DNA analogs are also very similar to that observed for tRNA (Paquette *et al.* 1990). DNA analogs of tRNA are aminoacylated by the cognate aminoacyl-tRNA synthetases, albeit at a much reduced rate (Kahn & Roe 1988). However, the 76 oligonucleotide DNA analogs of tRNAs are very large for complete physiochemical studies of metal ion coordination.

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Short DNA oligonucleotides that are analogs of tRNA stem and loops have been used to investigate the physical, chemical and biological properties of individual domains of tRNA molecules (Guenther *et al.* 1992, Dao *et al.* 1992, 1994). Modified nucleoside-containing hairpin structures can be designed and synthesized to study the binding of metals and subsequent conformational changes that could then modulate function. A single base methylation of cytosine in the DNA analog ($tDNA^{Phe}_{AC}$) of the yeast $tRNA^{Phe}$ anticodon stem greatly facilitates Mg^{2+} binding in the loop and results in a conformational transition of the loop (Dao *et al.* 1992). The m^5C-dependent, Mg^{2+}-induced structure of $tDNA^{Phe}_{AC}$–$d(m^5C)$ produces a stabilized double-stranded stem of five nucleotide complements. In addition, the seven-membered loop is closed by two base pairs resulting in a two-base turn. A 3′ stack begins in the loop and includes a single-base bulge (Dao *et al.* 1992, Guenther *et al.* 1992). The introduction of a second modified nucleoside, m^1G, positioned as in the tRNA anticodon loop, negates the Mg^{2+}-induced closed loop structure because m^1G is incapable of participating in a canonical base pair. The m^5C-dependent, Mg^{2+} stabilized, and m^1G open loop molecule was bound by programmed *Escherichia coli* 30S ribosomal subunits and effectively inhibited native $tRNA^{Phe}$ from binding (Dao *et al.* 1994). The higher-order, metal coordinating structures of the entire tDNA molecule and these smaller anticodon domains are potentially interesting models of DNA targets for highly site-specific metal carcinogens such as cadmium, nickel, chromium and arsenic. In this study we use circular dichroism (CD) spectroscopy to investigate the binding of various transition metals to DNA analogs of $tRNA^{Phe}$ and the conformational changes that result.

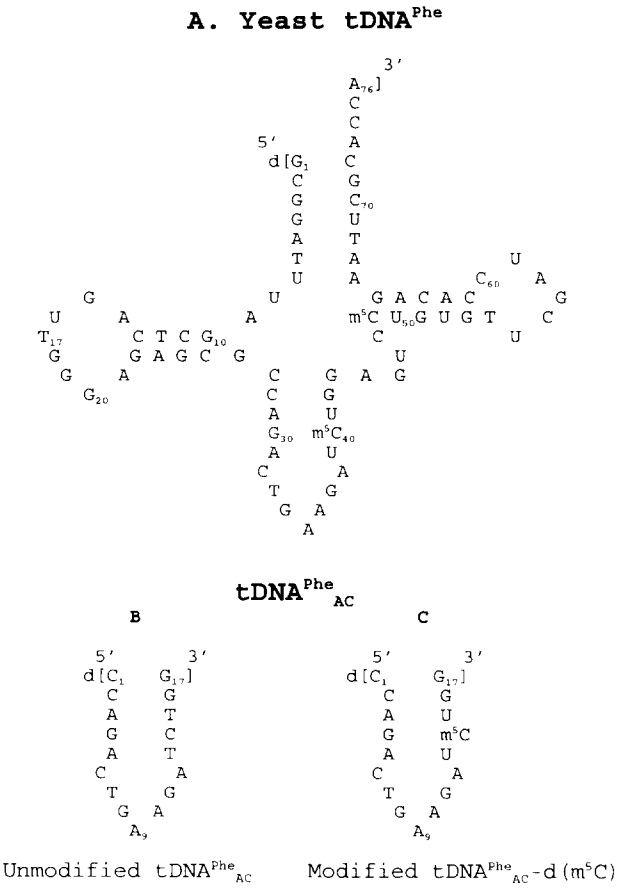

Figure 1. Sequence and secondary structure comparisons of the yeast $tRNA^{Phe}$, its anticodon stem/loop and DNA analogs. The molecules used in this study are DNA analogs of yeast $tRNA^{Phe}$ and its anticodon stem and loop domain (A–C). (A) The 76 deoxynucleotide DNA analog of yeast native $tRNA^{Phe}$, $tDNA^{Phe}$. (B) Unmodified DNA analog of the anticodon domain, $tDNA^{Phe}_{AC}$ with the sequence $d(T_{13}C_{14}T_{15})$ on the stem. (C) Modified $tDNA^{Phe}_{AC}$–$d(m^5C)$ containing the RNA-like sequence $d(U_{13}m^5C_{14}U_{15})$ on the stem.

Materials and methods

Preparation of $tDNA^{Phe}$, $tDNA^{Phe}_{AC}$ and $tDNA^{Phe}_{AC}$–$d(m^5C)$

DNA analogs of the yeast $tRNA^{Phe}$ anticodon stem and loop domain, $tDNA^{Phe}_{AC}$ and $tDNA^{Phe}_{AC}$ $d(m^5C)$ (Figure 1), were synthesized and purified according to published methods (Dao *et al.* 1992, Guenther *et al.* 1992). The entire 76 deoxynucleotide $tDNA^{Phe}$ (Figure 1) was synthesized in cooperation with BioSynthesis (Lewisville, TX). The base sequence was that of unmodified yeast $tRNA^{Phe}$ but composed of d(A, U, C, G) except at positions 6, 12, 17, 33, 54 and 68 where dTs were incorporated and positions 40 and 49 where $d(m^5C)$ was introduced. In $tRNA^{Phe}$, riboT occurs only at position 54 and m^5C at positions 40 and 49. The concentrations of the synthesized tDNAs in H_2O were determined by UV absorbance (HP 8451A diode array spectrophotometer) at 260 nm (1 A_{260} ml^{-1} equal to 50 $\mu g\ ml^{-1}$).

Transition metal preparation

The metal ions used in the studies included: Cd^{2+}, Pb^{2+}, Cu^{2+}, Co^{2+}, Cr^{2+}, Ni^{2+}, VO^{2+} and Zn^{2+}. The following salts were used: chloride for Pb^{2+} and Co^{2+}; sulfate for Cu^{2+}, VO^{2+} and Zn^{2+}; acetate for Cd^{2+}; and nitrate for Cr^{2+}. Ni^{2+} from NiO was dissolved using nitric acid. Metal ion stock solutions were 50 mM in deionized, 18 mΩ H_2O. To avoid diluting the tDNA sample when titrating with the metal ions, the appropriate volumes of metal ion stock solution were lyophyllized in Eppendorf tubes using a speed-vac concentrator and then dissolved in the tDNA solution as needed (Dao *et al.* 1992).

CD spectropolarimetry

CD spectra were collected with a Jasco J600 CD spectropolarimeter-interfaced IBM PC/2 microcomputer. CD spectra were monitored in the range of 190–350 nm and are reported in θ (mdeg). Temperature was maintained at 15 C for $tDNA^{Phe}_{AC}$, $tDNA^{Phe}_{AC}$–$d(m^5C)$ and $tDNA^{Phe}$ by placing the sample in a 1 ml cylindrical sample cell with an external water-circulating jacket.

Binding constants

The binding constants for the following chemical reaction:

$$n(\text{tDNA}) + m(\text{divalent metal}) \rightarrow \text{hairpin–metal complex} \quad (1)$$

can be expressed as:

$$K^* = [\text{tDNA hairpin}]^n[\text{cadmium}]^m/[\text{complex}] \quad (2)$$

The stoichiometric values for binding were determined by the Beltran-Porter method (Beltran-Porter *et al.* 1983) using titration data obtained over a range of substrate and ion concentrations. The determination of stoichiometry helps to calculate the binding constants using equation (2).

Gel electrophoresis

Samples of modified and unmodified $tDNA^{Phe}_{AC}$ were subjected to native and denaturing polyacrylamide gel electrophoresis (PAGE) (Grierson *et al.* 1982) to assess the possibility of metal ion catalyzed cleavage of phosphodiester bonds. The $tDNA^{Phe}_{AC}$ samples analyzed by PAGE had been treated with nano- and millimolar concentrations of transition metal ions under the same conditions as that for CD. Sample migration in the gel was detected by ethidium bromide staining. Untreated tDNA acted as a control for possible metal ion cleavage.

Results

Mg^{2+} and Cd^{2+} interactions with 76 nucleotide $tDNA^{Phe}$

In order to study the binding of metal ions to higher-order nucleic acid structures lacking the 2'OH, the entire 76 nucleotide DNA analog of yeast $tRNA^{Phe}$, $tDNA^{Phe}$, and the 17 nucleotide anticodon domain, $tDNA^{Phe}_{AC}$, were produced by automated chemical synthesis with site-specific nucleoside modifications (Figure 1). The CD spectrum of 76mer $tDNA^{Phe}$ had a wavelength maximum (λ_{max}) at 276.6 nm and a minimum (λ_{min}) at 243.4 nm, and was significantly effected by the addition of Mg^{2+} (Figure 2A). With the addition of Mg^{2+}, the ellipticity at λ_{max} increased by 15% and shifted to 274,6 nm. CD spectral changes observed with the addition of Mg^{2+} to $tDNA^{Phe}$ and the 17mer anticodon domain $tDNA^{Phe}_{AC}$ were very similar, i.e. the ellipticity at λ_{max} increased and λ_{max} was shifted to a lower wavelength (Figure 2B). By comparing difference spectra of $tDNA^{Phe}$ and $tDNA^{Phe}_{AC}$ generated from spectra in the absence and presence of Mg^{2+}, the degree to which the anticodon domain contributed to metal binding by the entire tDNA could be assessed. First, the $tDNA^{Phe}$ and $tDNA^{Phe}_{AC}$ spectra were normalized for their respective DNA concentrations. Then, difference spectra were produced by an arithmetic subtraction of the spectrum in the absence of Mg^{2+} from that in its presence. The difference spectrum for the 17mer $tDNA^{Phe}_{AC}$ was comparable to that of the 76mer $tDNA^{Phe}$ (Figure 2C). Thus, a substantial amount of change in the spectrum of $tDNA^{Phe}$ could be attributed to the tight metal ion binding by the anticodon alone.

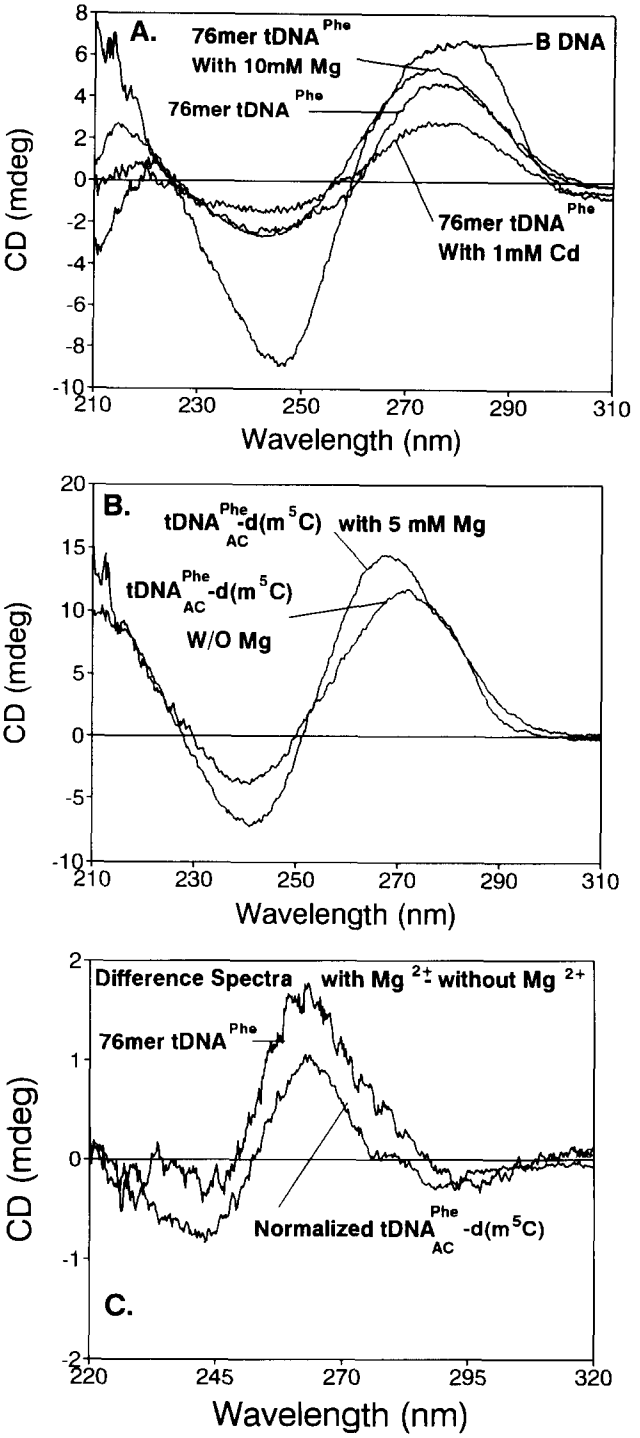

Figure 2. (A) CD spectra of the entire 76 nucleotide $tDNA^{Phe}$ and $tDNA^{Phe}_{AC}$. The spectrum of $tDNA^{Phe}$ was changed by the addition of Mg^{2+}. In the presence of Mg^{2+}, there was an increase in ellipticity and a shift away from the 280 nm maximum of B-DNA. In contrast to the interaction of Mg^{2+}, Cd^{2+} decreased the magnitude of the ellipticity at λ_{max} and shifted it toward 280 nm maximum of B-DNA. (B) Changes in CD spectra observed for the anticodon are very characteristic of those observed with the whole molecule. The presence of Mg^{2+} increased ellipticity and shifted the λ_{max} to a lower wavelength. (C) Difference spectra for 76mer $tDNA^{Phe}$ and $tDNA^{Phe}_{AC}$ in the presence or absence of Mg^{2+}.

Cd^{2+} was chosen as representative of divalent transition metals with the potential to interact with $tDNA^{Phe}$. Cd^{2+} has the utility of being an NMR resonant nucleus for future experiments. The effect of Cd^{2+} on the $tDNA^{Phe}$ CD spectrum differed markedly from that of Mg^{2+} (Figure 2A). A 1 mM concentration of Cd^{2+} decreased the 76mer $tDNA^{Phe}$ spectral ellipticity at λ_{max} and shifted λ_{max} to a slightly higher wavelength (Figure 2A). Cd^{2+} had a similar effect on the $tDNA^{Phe}_{AC}$–$d(m^5C)$ spectrum, but to a greater degree. The λ_{max} and λ_{min} were shifted to higher wavelengths and the ellipticities at both collapsed (Figure 3A).

Characterization of transition metal binding to $tDNA^{Phe}_{AC}$

The addition of a 1 mM concentration of each of the transition metal ions Pb^{2+}, Co^{2+}, Cr^{2+}, Ni^{2+}, VO^{2+}, Cu^{2+} or Zn^{2+} to $tDNA^{Phe}_{AC}$–$d(m^5C)$ effected the CD spectrum differently than Mg^{2+}, but similarly to Cd^{2+} (Figure 3A and B). The 1 mM addition of different transition metals to $tDNA^{Phe}_{AC}$–$d(m^5C)$ produced a general reduction of ellipticity at λ_{max}, the wavelength indicative of base stacking interactions. A reduced ellipticity in the range of 260–280 nm was indicative of diminished base stacking and, therefore, a loss of structural stability. Also, addition of transition metals produced a substantial and highly reproducible shift in λ_{max} toward the 280 nm maximum normally associated with B-form DNA (Figure 2A).

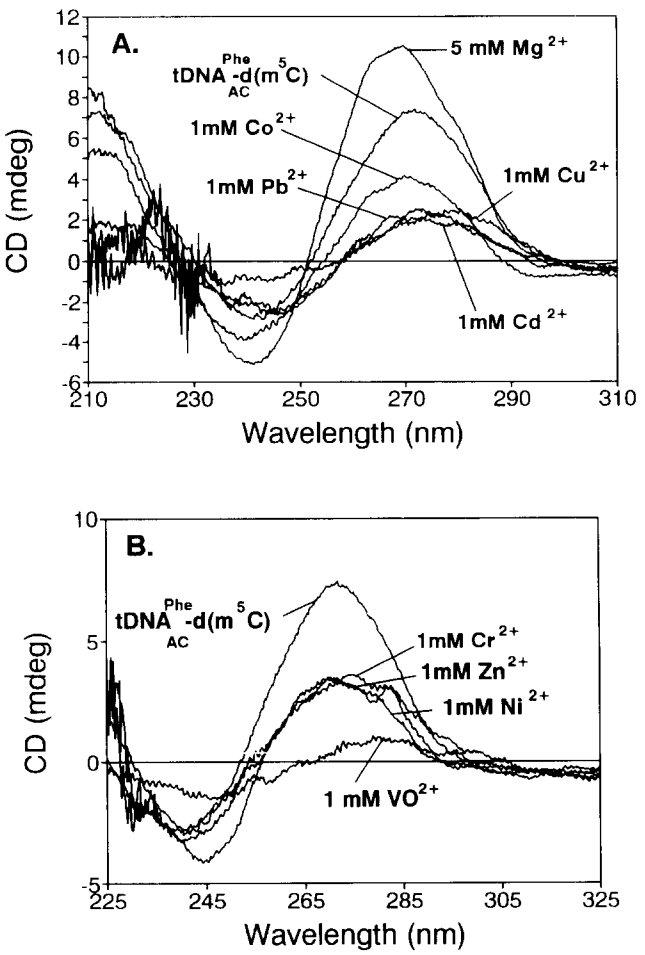

Figure 3. Effect of various heavy metal ions on the CD of $tDNA^{Phe}_{AC}$ $d(m^5C_{40})$. The CD spectra of $tDNA^{Phe}_{AC}$ $d(m^5C_{40})$ in the presence and absence of 5 mM Mg^{2+} are shown in contrast to the effect of 1 mM concentrations of: (A) Cd^{2+}, Co^{2+}, Pb^{2+}, Cu^{2+}; and (B) VO^{2+}, Cr^{2+}, Zn^{2+} and Ni^{2+}.

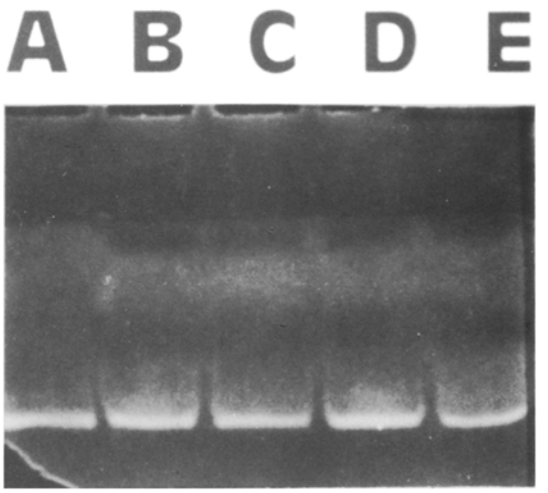

Figure 4. Gel electrophoresis analyses of the effect of metal ions on $tDNA^{Phe}_{AC}$ $d(m^5C_{40})$. To determine whether the effect of heavy metal ions on the CD spectrum of $tDNA^{Phe}_{AC}$–$d(m^5C_{40})$ were the result metal ion facilitated phosphodiester hydrolysis, treated and untreated $tDNA^{Phe}_{AC}$ $d(m^5C_{40})$ was subjected to denaturing PAGE: (A) untreated $tDNA^{Phe}_{AC}$ $d(m^5C)$; $tDNA^{Phe}_{AC}$–$d(m^5C)$ treated with (B) Cd^{2+}; (C) Co^{2+}; (D) Mg^{2+}; (E) VO^{2+}.

The decreased amplitude of the CD spectral maximum could have been the result of metal ion catalyzed hydrolysis of phosphodiester bonds. If cleaved, the nucleic acid structure would have generated small oligomers and mononucleotides that could be observed by an increase in UV absorbance. Under the conditions used for CD spectroscopy, neither transition metals nor Mg^{2+} produced hyperchromicity in the UV spectrum of $tDNA^{Phe}_{AC}$–$d(m^5C)$. This result does not preclude a small number of cleaved bonds in the nucleic acid backbone. Small numbers of cleaved phosphodiester bonds, including a single nick, could be detected by comparing the migrations of treated and untreated $tDNA^{Phe}_{AC}$–$d(m^5C)$ samples during native and denaturing PAGE. Untreated $tDNA^{Phe}_{AC}$–$d(m^5C)$ and samples treated with Mg^{2+} or with metal ions migrated similarly upon native and denaturing PAGE (Figure 4). Importantly, there was only one band of $tDNA^{Phe}_{AC}$ per sample and no variation in band intensity as would be expected if fragmentation occurred. Therefore, it is highly unlikely that the observed loss of spectral ellipticity in the spectrum of $tDNA^{Phe}_{AC}$–$d(m^5C)$ with addition of transition metal ions is due to the cleavage of phosphodiester bonds.

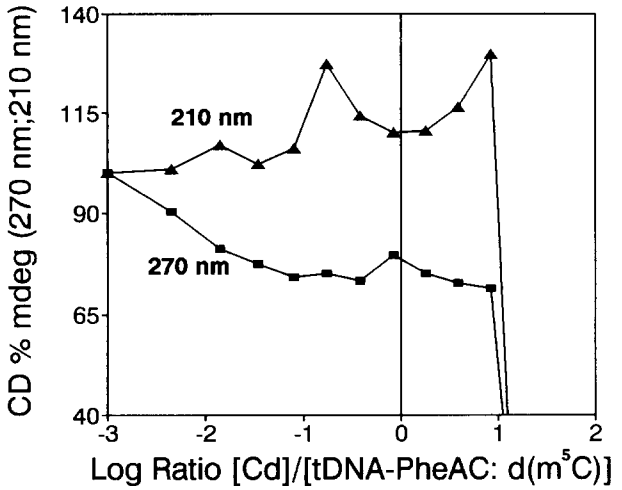

Figure 5. Titration of $tDNA^{Phe}_{AC}$–d(m^5C_{40}) with Cd^{2+}. The $tDNA^{Phe}_{AC}$–d(m^5C_{40}) was titrated with Cd^{2+} and the conformational transition monitored by CD at 270 and 210 nm. The percent change in the amplitude of ellipticity at the two wavelengths is plotted against the log of the ratio of [Cd^{2+}] to [$tDNA^{Phe}_{AC}$–d(m^5C_{40})].

Mg^{2+} and Cd^{2+} competition for the m^5C-dependent metal binding site in $tDNA^{Phe}_{AC}$

The $tDNA^{Phe}_{AC}$–d(m^5C) was titrated with Cd^{2+} over the concentration range of 20 nM to 2 mM. Structural alterations were monitored by CD at two wavelengths, 270 and 210 nm. Nanomolar concentrations of Cd^{2+} were sufficient to induce a conformational transition of $tDNA^{Phe}_{AC}$–d(m^5C) and, thus, alter the CD spectrum (Figure 5). Increased concentrations of Cd^{2+} resulted in two conformational transitions of $tDNA^{Phe}_{AC}$–d(m^5C). With increasing [Cd^{2+}], the ellipticity at λ_{270} continued to decrease until a ratio of [Cd^{2+}] to [$tDNA^{Phe}_{AC}$–d(m^5C)] of approximately 0.06 was reached. Between molar ratios of approximately 0.1 and 8.4, the ellipticity at λ_{270} hardly changed. Because the 270 nm contribution to the CD spectrum is correlated with base stacking interactions, the data indicated that a base stacked conformation different from that induced by Mg^{2+} (Dao *et al.* 1992) was obtained in the presence of Cd^{2+} at a [Cd^{2+}] to [$tDNA^{Phe}_{AC}$–d(m^5C)] ratio of 0.1–1.0. At molar ratios greater than 10, the spectrum deteriorated dramatically, indicating the denaturation of the structure. The behavior of the spectrum at the negative ellipticity of 210 nm was in contrast to the positive ellipticity at 270 nm. The ellipticity at λ_{210} continuously increased with an increase in the ratio of [Cd^{2+}] to [$tDNA^{Phe}_{AC}$] until the molar ratio of 8.4 was reached, and then dramatically declined (Figure 5). The 210 nm contribution to the CD spectrum is attributed to the backbone conformation. In this case, the backbone conformation must have been changed continuously until a molar ratio greater than 8.4 was reached, at which time the spectrum deteriorated and, thus, the molecular structure denatured. The primary Cd^{2+} dissociation constant was determined from titrations of $tDNA^{Phe}_{AC}$–d(m^5C) over a range of ion and tDNA concentrations and the data evaluated with the Beltran-Porter method (Beltran-Porter 1983, Chen *et al.* 1993)(Figure 6). The number of Cd^{2+} bound per tDNA was 1 at low Cd^{2+} to tDNA ratios; the K_d was 3.8×10^{-7} M.

In order to determine if the Mg^{2+}-induced $tDNA^{Phe}_{AC}$–d(m^5C) conformation was maintained when exposed to Cd^{2+}, $tDNA^{Phe}_{AC}$–d(m^5C) was titrated with Cd^{2+} in the presence of Mg^{2+}. The conformational transition of $tDNA^{Phe}_{AC}$–d(m^5C) produced by low molar ratios of [Cd^{2+}] to [$tDNA^{Phe}_{AC}$–d(m^5C)] in the absence of Mg^{2+} (Figure 5) was not observed in the presence of 20 mM Mg^{2+}. In fact, there was little change in the 270 nm ellipticity until the molar ratio of 39 was reached, when the spectrum deteriorated dramatically as it did in the absence of Mg^{2+}. The ellipticity at 210 nm increased slightly in reaching the molar ratio of 84 and then with increasing concentrations of Cd^{2+} decreased as significantly as detected in the absence of Mg^{2+}. Therefore, the Mg^{2+}-induced and stabilized $tDNA^{Phe}_{AC}$–d(m^5C_{40}) conformation had a higher tolerance to the effects of Cd^{2+} by a factor of 5–10 than $tDNA^{Phe}_{AC}$–d(m^5C) in the absence of Mg^{2+}.

Effect of Cd^{2+} on CD spectrum of unmodified $tDNA^{Phe}_{AC}$

Strong Mg^{2+} binding to $tDNA^{Phe}_{AC}$ requires a base methylation of cytosine in the anticodon stem (Guenther *et al.* 1992). This single modification plays a critical role in defining the strong Mg^{2+} binding site in the loop of the hairpin structure (Dao *et al.* 1992). The properties of Cd^{2+}, unlike that of Mg^{2+}, enabled the heavy metal ion to induce conformational transitions in both modified and unmodified $tDNA^{Phe}_{AC}$. However, in order to effect changes in the unmodified $tDNA^{Phe}_{AC}$ spectrum comparable to those of the

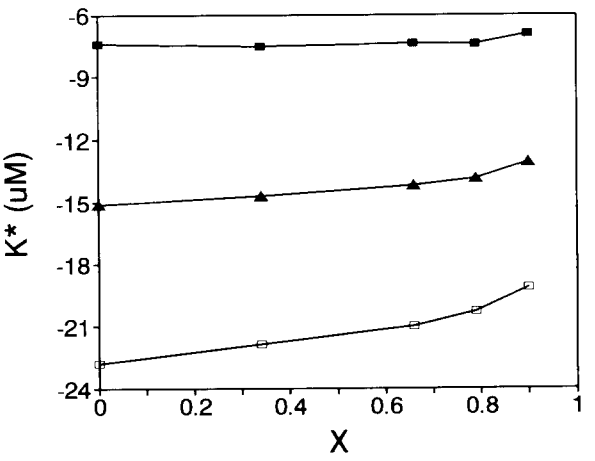

Figure 6. Determination of the stoichiometry of binding of Cd^{2+} to modified $tDNA^{Phe}_{AC}$–d(m^5C). For the Beltran-Porter determination of binding stoichiometry and binding constants, values of K^* are calculated and plotted against x, the fraction of total Cd^{2+}–$tDNA^{Phe}_{AC}$ complex for different concentrations of Cd^{2+}, and different values of m and n were used to calculate values of K^* (see Materials and methods). When the relative amounts of both Cd^{2+} and $tDNA^{Phe}_{AC}$ are kept at 1, a line with almost no slope results (closed squares). However, when the relative amount of Cd^{2+} is 2 or 3 and that of $tDNA^{Phe}_{AC}$–d(m^5C) is 1, the resulting lines (triangles and open squares, respectively) were sloped. The figures indicates that the binding ratio of Cd^{2+} to $tDNA^{Phe}_{AC}$–d(m^5C) is 1:1.

$tDNA^{Phe}_{AC}$–d(m^5C) spectrum, a higher concentration of Cd^{2+} was required. With increasing [Cd^{2+}], the ellipticity at λ_{270} decreased continually until a ratio of [Cd^{2+}] to [$tDNA^{Phe}_{AC}$] of 39 was reached. Between molar ratios of approximately 0.16 and 39, the ellipticity at λ_{270} decreased less rapidly than between 0 and 0.16. As with $tDNA^{Phe}_{AC}$ d(m^5C), the spectrum of unmodified $tDNA^{Phe}_{AC}$ deteriorated completely at molar ratios of [Cd^{2+}] to [unmodified $tDNA^{Phe}_{AC}$] higher than 39. The ellipticity at λ_{210} hardly changed until the [Cd^{2+}] to [$tDNA^{Phe}_{AC}$] ratio reached 39, then the amplitude collapsed as with $tDNA^{Phe}_{AC}$ d(m^5C). In contrast to the titration of $tDNA^{Phe}_{AC}$ d(m^5C), the titration of unmodified $tDNA^{Phe}_{AC}$ indicated a continually changing conformation and no hint of the existence of a stable intermediate conformation.

Discussion

CD spectroscopy is well suited for determining conformational changes in nucleic acids associated with the introduction of modified nucleodides and or metal ion coordination (Guenther *et al.* 1992, Dao *et al.* 1992, Chen *et al.* 1993). The A, B and Z forms of DNA have unique CD spectra that can be compared with spectra of DNAs of known sequence, but unknown structure. Thus, the CD spectra of the 76 oligonucleotide $tDNA^{Phe}$ (Figure 2) in the presence or absence of Mg^{2+} indicated that the overall form of this $tDNA^{Phe}$ analog was neither A-, B- nor Z-DNA. Likewise the global conformation taken by the $tDNA^{Phe}_{AC}$–d(m^5C) domain, with or without metal ion present, was not determinable from CD alone. However, preliminary NMR analyses has indicated that within the m^5C-dependent, Mg^{2+}-induced conformation 14 deoxynucleosides are in the 2'endo sugar conformation characteristic of B-DNA, and the remaining three are 3'endo (Sierzputowska-Gracz & Agris, unpublished results). We believe that the 3'endo puckers and unconventional loop structure imposed by Mg^{2+} coordination and two intra-loop base pairs are responsible for the CD spectrum of $tDNA^{Phe}_{AC}$–d(m^5C) being different from that of B-DNA.

The conformation of the $tDNA^{Phe}$ molecule was sensitive to the presence of magnesium (Figure 2A). Most of the changes in CD could be assigned to the anticodon domain of tDNA because d(m^5C) is the dominant feature necessary for metal-binding (Figure 2C). This fact had allowed us to narrow our focus and choose $tDNA^{Phe}_{AC}$–d(m^5C) as an appropriate model of transition metal binding. $tDNA^{Phe}_{AC}$–d(m^5C) has a surprisingly strong inner-sphere metal binding site (Mg^{2+} $K_d = 1.09 \times 10^{-6}$ M) and concomitant structural transition to a higher-order conformation (Guenther *et al.* 1992, Dao *et al.* 1992). Mg^{2+} is not necessarily the only divalent cation that could possibly be attracted to the strong binding site of the anticodon. Heavy metal ions exhibited significant effects on $tDNA^{Phe}_{AC}$–d(m^5C) conformation (Figure 3). The metal ions caused a highly reproducible shift of the CD spectrum toward the 280 nm maximum normally associated with B-form DNA. In general, heavy metals with the larger ionic radii shifted λ_{max} of $tDNA^{Phe}_{AC}$ d(m^5C) more than ions with smaller radii. For example, at 1 mM concentrations Cd^{2+}, with an ionic radius of 0.97 Å, shifted λ_{max} more than Co^{2+} with a 0.72 Å radius. Significantly, the transition metals caused a collapse of ellipticities at λ_{max} and λ_{min}. The metal ions facilitated a conformational transition of the $tDNA^{Phe}_{AC}$ toward a denatured state, but did not do so by catalyzing cleavage of the phosphodiester backbone. Cd^{2+} denatured the $tDNA^{Phe}_{AC}$–d(m^5C) structure at nanomolar concentrations.

The octrahedral coordination of Cd^{2+} with favored O–N ligands common to DNA, its future utility in NMR and known environmental hazards have made Cd^{2+} worthy of additional focus. Detailed investigation on $tDNA^{Phe}_{AC}$ interaction with Cd^{2+} revealed that at low ratios of Cd^{2+} to $tDNA^{Phe}_{AC}$, the $tDNA^{Phe}_{AC}$–d(m^5C) structure formed a relatively less stable intermediate conformation before being denatured at higher Cd^{2+} concentrations. Nanomolar concentrations of Cd^{2+} were sufficient to alter the $tDNA^{Phe}_{AC}$ d(m^5C) in a manner different from that produced by micromolar Mg^{2+}. The tight cadmium binding requires the presence of d(m^5C) within the stem of the molecule. The resulting structure could be in a state of partial denaturation. In the presence of 20 mM Mg^{2+}, higher concentrations of Cd^{2+} were required to denature $tDNA^{Phe}_{AC}$–d(m^5C) and the intermediate conformation was not observed. The data indicate that d(m^5C) played a critical role in altering ligand presentation for Mg^{2+} and Cd^{2+} binding. The CD data strongly suggest that Cd^{2+}, having the lower K_d by a factor of 3, competes effectively for the tight Mg^{2+} binding site within the loop of $tDNA^{Phe}_{AC}$–d(m^5C) (Dao *et al.* 1992). Cd^{2+} directly coordinated to the DNA ligands; whereas, Mg^{2+} most likely enters the DNA binding site as a hydrate (Basti & Agris, personal communication).

CD spectra at 210 and 270 nm suggest that a two-step mechanism was involved in Cd^{2+} denaturation of $tDNA^{Phe}_{AC}$ d(m^5C). First, Cd^{2+} has a very high binding affinity for the d(m^5C)-dependent $tDNA^{Phe}_{AC}$ conformation and will bind in preference to Mg^{2+}, displacing the functionally essential metal. However, we found that the Mg^{2+}-induced structure of $tDNA^{Phe}_{AC}$–d(m^5C) was retrievable with addition of Mg^{2+} while in this intermediate form. The second step is a cooperative event with the initially bound Cd^{2+} facilitating the binding of other Cd^{2+} ions. Simultaneously, the diphosphodiester backbone is denatured by Cd^{2+} binding with a low affinity and in a non-specific manner. The multiple ion binding significantly deteriorates structural stability. The structure cannot be restored by the addition of Mg^{2+}. Therefore, a single base methylation of cytosine in the anticodon stem of $tDNA^{Phe}$ plays an important role in metal binding and conformation.

Titration of the unmodified $tDNA^{Phe}_{AC}$ did not reveal an intermediate conformation. Since, the unmodified $tDNA^{Phe}_{AC}$ does not have the d(m^5C)-dependent metal binding site, ion binding progressed similarly to the second step of the Cd^{2+} induced $tDNA^{Phe}_{AC}$–d(m^5C) interaction. The data suggest that Cd^{2+} binding to unmodified $tDNA^{Phe}_{AC}$ involved multiple ion binding that perturbed the backbone and resulted in a denatured structure. Because Cd^{2+} was bound to

unmodified $tDNA^{Phe}_{AC}$ with a relatively weak, non-specific affinity increasing concentrations of Mg^{2+} could displace bound Cd^{2+}. Unmodified $tDNA^{Phe}_{AC}$ denatured by Cd^{2+} could be rescued by increasing Mg^{2+}.

Besides defining the strong Mg^{2+} binding site, the single cytosine methylation of $tDNA^{Phe}_{AC}$ facilitated a heavy metal ion-induced structural transition toward a denatured conformation. We hypothesize that Cd^{2+} and perhaps other transition metal ions substitute for Mg^{2+} at the tight Mg^{2+} binding site in the loop and, because of their larger radii, induce a conformational transition different from that in the presence of Mg^{2+}. The strong binding of heavy metals, particularly Cd^{2+} to DNA structures may have important biological significance. The toxic effects from acute exposure to many metals has long been known. More recent investigations have focused on hazards related to long-term chronic exposure. In addition to metals being causative agents in a number of diseases, exogenous metals are being recognized as potentially important carcinogens (Kraybill & Mehlman 1977, Waalkes *et al.* 1992). Clinical research has confirmed nickel, chromium and arsenic as definite human carcinogens. Similar to these carcinogens, Cd^{2+} possesses remarkable target site specificity that is noted by low concentration and tight binding to a specific region within the tDNA hairpin.

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