Nucleic acid duplex stability: influence of base composition on cation effects

Shu-ichi Nakano¹, Mariko Fujimoto¹, Hideyuki Hara¹ and Naoki Sugimoto¹,2,*

¹Department of Chemistry, Faculty of Science and ²High Technology Research Center, Konan University, 8-9-1 Okamoto, Higashinada-ku, Kobe 658-8501, Japan

Received February 21, 1999; Revised and Accepted June 3, 1999

ABSTRACT

The effects of counter ion on a nucleic acid duplex stability were investigated. Since a linear free energy relationship for the thermostability of oligonucleotide duplexes between those in 1 M and in 100 mM NaCl-phosphate buffer were observed regardless of whether they are DNA–DNA, RNA–RNA or RNA–DNA duplexes, simple prediction systems for $\Delta G^\circ$, as well as $T_m$ values in 100 mM NaCl-phosphate buffer were established. These predictions were successful with an average error of only 2.4°C for $T_m$ and 5.7% for $G^\circ$ values. The number of Na⁺ newly bound to a duplex when the duplex forms (−Δ$n$) was significantly influenced by the base composition, and −Δ$n$ for d(GCATGC)₂ (16), r(AUGCAU)₂ with 3’ dangling end (17), or nearest-neighbor base pairs for the cation binding, almost no additive effects on the duplex stability was observed for NaCl and MgCl₂, suggesting a competitive binding for these cations. The sequence-dependent manner of Δ$n$ suggests the presence of preferential base pairs or nearest-neighbor base pairs for the cation binding, which would affect nearest-neighbor parameters.

INTRODUCTION

The stability of many kinds of nucleic acid duplexes were measured to investigate their stable folding structures, potential as recognition sites by a protein, as candidate target sites for an antisense nucleotide, etc. (1–3). Now, it is possible to estimate the thermodynamics for oligonucleotide duplexes by nearest-neighbor parameters, and we can easily evaluate any duplex stability with good agreement with experimental results (4–9). However, since all of these prediction parameters are determined in 1 M NaCl-phosphate buffer, they are ambiguous for the prediction of duplex stabilities under physiological salt conditions, e.g., 100 mM NaCl and NaCl with MgCl₂. Despite the importance of the salt concentration and species on the duplex thermostability, detailed experimental data are lacking, though some information for polynucleotides of different base composition were reported described as a melting temperature ($T_m$) (10). Since the $T_m$ for a polynucleotide is the temperature at which 50% of the base pairs in a duplex have been dissociated (11), it is difficult to determine the number of dissociated base pairs and to estimate the effects of loops and single-stranded nucleotides on duplex stability. They also had less possibilities for forming secondary structures such as hairpin loops, thereby lowering the observed duplex stability (12). There are publications about salt effect on oligonucleotide duplex stability of d(AT) oligomers (13), r(AGUAUA)₂ (14), dumb-bell shaped DNAs (15), d(GCATGC)₂ (16), r(AUGCAU)₂, with 3’ dangling end (17), parallel-stranded DNA (18) etc. However, systematic information about nucleic acid hybridization and stability data also exist. Lesnik and Freier reported $T_m$ and $\Delta G^\circ$ data in 100 mM NaCl-phosphate buffer (19,20) though they used relatively longer oligonucleotides, up to 21 bp, and values were estimated from only a single melting curve. To determine quantitative salt effects on a nucleic acid duplex, reliable thermodynamic data on short oligonucleotides are needed. Here, we used short (14 bp) oligonucleotide duplexes that dissociate completely to become separated strands at temperatures higher than the $T_m$ value and that can be applied to the nearest-neighbor model for the duplex stability (4–9). We measured thermodynamics in 100 mM NaCl-phosphate buffer for 30 kinds of oligonucleotide duplexes including those DNA–DNA, RNA–RNA and RNA–DNA composition, and developed a prediction system for their stabilities. Furthermore, several monovalent and divalent cations were also investigated. With these results, we described physical roles for cations on a duplex stability in comparison to reported information about cations with nucleotides arising from ab initio, semi-empirical and empirical results.

MATERIALS AND METHODS

Materials

All oligodeoxyribonucleotides and oligoribonucleotides were synthesized chemically on a solid support using phosphoramidite procedures and purified with reversed-phase high performance liquid chromatography (HPLC) after deblocking operations. These nucleotides were desalted with a C-18 Sep-Pak cartridge. All of these oligonucleotide duplexes were designed to be from 6 to 14 nt long and to form Watson–Crick base pairs without producing unpaired nucleotides nor a hairpin-looped structure. Oligonucleotide concentration was determined from the absorbance at 260 nm with single strand extinction coefficients calculated from mononucleotide and dinucleotide data of a nearest-neighbor approximation (21). All duplexes, DNA
duplex (DNA–DNA), RNA duplex (RNA–RNA) and RNA–DNA hybrid duplex (RNA–DNA), were prepared by mixing an equimolar concentration of DNA and RNA strands.

**UV measurements**

Absorbance measurements were made on Hitachi U-3200, U-3210 and Beckman DU 640 spectrophotometers. Melting curves (absorbance versus temperature curves) were measured at 260 nm with a connected temperature controller. The water condensation on the cuvette exterior at a low-temperature range was avoided by flushing with a constant stream of dry N₂ gas. The heating rate was 0.5 or 1.0°C/min. UV melting curves were collected in NaCl-phosphate buffer, which contains NaCl, 10 mM Na₂HPO₄ and 1 mM Na₂EDTA (pH 7.0). Cation chloride-cacodylate buffers were prepared by dissolving each cation chloride in 10 mM sodium cacodylate solution to the desired cation chloride concentration; thereafter the pH value was adjusted to 7.0.

**Determination of thermodynamics for duplex formations**

Melting curves were fitted with a procedure to obtain thermodynamic parameters (ΔH°, ΔS° and ΔG°₃₇) for a double helix formation as described elsewhere (22). This method makes an estimation of the thermodynamic values from the shape of each melting curve. To increase the accuracy of these determinations, we also evaluated them from plots of Tₘ⁻¹ versus ln(C/s). These thermodynamic values were also analyzed by the following equations (5,6):

\[ T_m^{-1} = R \cdot \ln(C/s) / \Delta H^° + \Delta S^° / \Delta H^° \]  

\[ \Delta G^°₃₇ = \Delta H^° - 310.15 \cdot \Delta S^° \]

where Cₜ is the total strand concentration, and s reflects a sequence symmetry of the self (s = 1) or non-self complementary strands (s = 4).

Estimated errors for thermodynamic values (σₖ,σₛ and σₖₛ₃₇) derived from a curve fitting procedure were the standard deviations among data points of each melting curves measured at different Cₜ. Those for ΔH° and ΔS° (σₖ,σₛ₃₇) from Tₘ⁻¹ versus ln(C/s) plots were estimated from the linearity of the plots and those for ΔG°₃₇ (σₖₛ₃₇) were calculated by the following equation:

\[ (σₖₛ₃₇)^2 = (σₖ)^2 + 310.15^2 (σₛ)^2 - 2 \cdot 310.15 \cdot (R \cdot σₖ \cdot σₛ) + σₖ \cdot σₛ \]  

where Rₖ, σₛ are the correlation coefficient between ΔH° and ΔS° (8,9,23). The final thermodynamic parameters were evaluated from the average values obtained from the curve fitting and Tₘ⁻¹ versus ln(C/s) plots.

**Prediction of thermodynamics by nearest-neighbor parameters**

Nearest-neighbor parameters for the nucleic acid duplexes were reported by Sugimoto et al. (6) and SantaLucia Jr et al. (7,8) for DNA–DNA (DNA duplex), Freier et al. (4) and Xia et al. (9) for RNA–RNA (RNA duplex) and Sugimoto et al. (5) for RNA–DNA hybrid duplexes. According to the nearest-neighbor model, thermodynamic values of ΔH°, ΔS° and ΔG°₃₇ for a duplex formation consists of the following three terms: (i) a free energy change for a helix propagation as a sum of each subsequent base pair, 10 kinds of data sets for both DNA–DNA and RNA–DNA but 16 sets for RNA–DNA (5); (ii) a free energy change for the helix initiation; and (iii) a free energy change of a mixing entropy term when a duplex is composed of a self-complementary strand. The sequences of oligonucleotide duplexes were divided into nearest-neighbor base pairs and these three terms were summed up according to the nearest-neighbor parameters (4–9). Melting temperatures (Tₘ values) at 8 µM total nucleotide strand concentration were calculated with predicted ΔH° and ΔS° from the equation 1.

**Circular dichroism (CD) measurements**

CD spectra were obtained on a JASCO J-600 spectropolarimeter equipped with a temperature controller. The experimental temperature was 5°C. The cuvette-holding chamber was flushed with a constant stream of dry N₂ gas to avoid water condensation on the cuvette exterior. All CD spectra were measured from 320 to 200 nm in 0.1 cm path-length cuvettes. The concentration of the samples was 70 µM in 100 mM NaCl/10 mM phosphate/1 mM Na₂EDTA (pH 7.0) or 1 mM MgCl₂/10 mM sodium cacodylate buffer (pH 7.0).

**RESULTS AND DISCUSSION**

**Thermodynamics of nucleic acid duplex in 100 mM NaCl-phosphate buffer**

Predictions of the stability of nucleic acid duplexes (DNA–DNA, RNA–RNA and RNA–DNA) in 1 M NaCl concentration are successful using nearest-neighbor parameters (4–9). To estimate the duplex stabilities in biological salt conditions, an extended usage of these nearest-neighbor parameters for the stability predictions in 100 mM NaCl was examined. Prediction of the duplex stability in 100 mM NaCl buffer is badly needed in view of the usage of antisense oligonucleotides in biological systems. Thermodynamics (ΔH°, ΔS° and ΔG°₃₇) for 30 kinds of oligonucleotide duplexes (21 DNA–DNA, 6 RNA–RNA and 3 RNA–DNA duplexes) in 100 mM NaCl-phosphate buffer were measured and their parameters are listed in Table 1 including Tₘ values at 8 µM. All of these duplexes showed a two-state transition in 1 M NaCl-phosphate buffer and their thermodynamics in 1 M NaCl were predicted by nearest-neighbor parameters with a high accuracy. Here, all of these duplexes also showed a two-state transition in 100 mM NaCl with <10% differences among the parameters determined by curve fittings and Tₘ⁻¹ versus ln(C/s) plots. As expected, these duplexes decreased their stability with respect to those measured in 1 M NaCl (5,6) because of the anionic character of the nucleotide. Three DNA duplexes of d(GACTAGTC)₂, d(GAGTACTC)₂ and d(GTCTAGAC)₂ having the same nearest-neighbor base pairs presented similar thermodynamic parameters, indicating the nearest-neighbor model can also be applied in 100 mM NaCl. We employed a relationship between thermodynamics in 100 mM NaCl and those predicted for 1 M NaCl are given in Figure 1A, including those determined by Lesnik and Freier, although they used relatively longer (more stable) oligonucleotides and estimated Tₘ and ΔG°₃₇ values from the shape of a single melting curve (19,20). They showed good correlations of Tₘ values obtained in 100 mM with their predicted values. For ΔG°₃₇, linear plots were also found as shown
Table 1. Thermodynamic parameters of helix formation in NaCl-phosphate buffer determined with $T_m$ versus $\ln C_t$ and curve fitting procedure $^a$

<table>
<thead>
<tr>
<th>Sequence</th>
<th>$\Delta H^c / \text{ kcal mol}^{-1}$</th>
<th>$\Delta S^c / \text{cal mol}^{-1} \text{K}^{-1}$</th>
<th>$T_m^b / ^\circ\text{C}$</th>
<th>$\Delta H^\circ / \text{ kcal mol}^{-1}$</th>
<th>$\Delta S^\circ / \text{cal mol}^{-1} \text{K}^{-1}$</th>
<th>$T_m^\circ / ^\circ\text{C}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA/DNA duplex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\text{dCC} \rightarrow \text{dG}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\text{dAA} \rightarrow \text{dTT}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA/RNA duplex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\text{nAGUAGUA(U)}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\text{nGUA(U)}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNA/DNA duplex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$All experiments were done in buffer containing 100 mM NaCl/10 mM Na$_2$HPO$_4$/1 mM Na$_2$EDTA, pH 7.0.

$^b$Melting temperatures were calculated at the total oligomer strand concentration of 8 µM.
in Figure 1B. These linear plots were fitted to a straight line, giving the following equations:

\[
T_m (100 \text{ mM}) = 0.876 T_m (1 \text{ M}) - 5.148 \tag{4}
\]

\[
\Delta G^{37} (100 \text{ mM}) = 0.630 \Delta G^{37} (1 \text{ M}) - 1.667 \tag{5}
\]

These equations indicate the possibility of estimating \(T_m\) and \(\Delta G^{37}\) values in 100 mM NaCl by using nearest-neighbor parameters determined in 1 M NaCl. Equation 5 indicates a linear free energy relationship (LFER) between \(\Delta G^{37}\) values in 1 M and in 100 mM NaCl, and when the other prediction parameters reported by Allawi and SantaLucia for DNA duplex (8) and Xia et al. for RNA duplex (9) were applied, almost the same equation of \(\Delta G^{37} (100 \text{ mM}) = 0.632 \Delta G^{37} (1 \text{ M}) - 1.360\), was obtained. Consequently, \(T_m\) and \(\Delta G^{37}\) values in Table 1 can be predicted by these equations with an average error of 4.4°C and 5.7%. These are improved estimates with regard to previously reported methods (those of 3.7°C and 8.6%, respectively) which emphasize the number of phosphate groups of a duplex on the salt effect (7,24). These equations are also used to predict another 86 data sets that have been measured by several groups (19,20,25,26). The resulting average errors of the predictions for \(T_m\) and \(\Delta G^{37}\) are 3.8°C and 10.2%, respectively. The equations 4 and 5 agree well with the relatively shorter (unstable) nucleotides, but less well with the longer (stable) ones. This might be because of a non-two-state transition or a limitation of the nearest-neighbor model for some longer nucleotides examined by Lesnik and Freier (19,20). Lesnik and Freier also reported linear relationships of \(T_m\) and \(\Delta G^{37}\) in 100 mM NaCl versus predicted \(T_m\) and \(\Delta G^{37}\) in 1 M NaCl for RNA duplex and for RNA–DNA hybrid duplex separately (20). Here, we suggest the equations 4 and 5 regardless of DNA–DNA, RNA–RNA or RNA–DNA duplex.

Quantitatively, effects of the sodium ion concentration on the thermostability of nucleic acid duplex are considered with the following equilibrium relations (27,28):

\[-\alpha \Delta n = \frac{\partial \log K}{\partial \log [Na^+]}\]

\[
(d \frac{T_m}{d \log [Na^+]} / 2.303 = \alpha \Delta n R T_m / 2H^o\]

where \(\alpha\) correlates with an activity coefficient of the sodium ion (\(\gamma\), equal to \((1 + d \log [Na^+] / d \log [Na^+]])\) and which is regarded as a constant of 0.92 over the NaCl concentration range considered here (17,28,29). \(\Delta n\) is the number of sodium ions released from a duplex in the duplex formation, resulting in a negative \(n\) value for a duplex formation (28). The association constant of \(K_a\), \(\Delta H^o\) (enthalpy change per mole of nucleotide) and \(T_m\) values can be obtained from the measurements of duplex stability. The linear correlation observed for \(\Delta G^{37}\) values between 1 M and 100 mM NaCl in spite of nucleotide sequence, chain length and duplex species (Fig. 1B) means any two duplexes with the same stability show the same \(d \log K / d \log [Na^+]\) value suggested from equation 5, resulting in the same number of \(Na^+\) required for duplex formation given in equation 6. Thus, \(\Delta n\) for \(Na^+\) would be significantly affected by the base composition as well as the number of phosphate groups.

**Correlation among thermodynamics for nucleic acid duplex formations**

Figure 2A shows a relationship between \(T \Delta S^o\) and \(\Delta H^o\) for 204 duplex formations, 87 DNA–DNA (6,7), 49 RNA–RNA (4,11,30) and 68 RNA–DNA (5) measured in 1 M NaCl phosphate buffer. Although a rectangular hyperbola relationship between them has been suggested (31), a good straight line (\(T \Delta S^o = 0.868 \Delta H^o - 0.304\)) with a correlation coefficient of \(r^2 = 0.99\) was revealed due to a compensation between \(\Delta H^o\) and \(\Delta S^o\). It is considered that the slope of the plot is attributable to the structural demands necessary for the nucleotide association, and that of the intercept is an entropy contribution owing to the release of solvent when a complex forms (32). For \(T \Delta S^o\) and \(\Delta H^o\) plots for porphyrin derivatives associated with quinoline analogs showed 0.62 and 0.11 kcal mol\(^{-1}\) for the slope and its intercept, respectively (32). A larger slope for oligonucleotides than for porphyrin with quinoline is reasonable in view of the flexible structure of nucleotides; however it is unlikely to explain the negative intercept for oligonucleotides because water molecules dissociated from nucleotides result in a positive entropy change upon a duplex formation. Thus, the negative
intercept of Figure 2A indicates that an association such as cation bindings to a duplex should be incorporated for the nucleotide duplex formation. This suggests a role of cation in the duplex initiation, which also reflects to a helix initiation factor in the nearest-neighbor parameter (6).

$T_m$ and $\Delta G^\circ_{37}$ values measured in both 1 M and 100 mM NaCl buffer also revealed a linear correlation between reciprocals of $T_m$ and those of $-\Delta G^\circ_{37}$. The plots in Figure 2B were grouped into two straight lines depending on whether a self-complementary sequence of the duplex was involved. With these two linear equations of $T_m^{-1} = -3.36 \times 10^{-3} \cdot \Delta G^\circ_{37}^{-1} + 2.81 \times 10^{-3}$ for self complementary strands and $T_m^{-1} = -3.27 \times 10^{-3} \cdot \Delta G^\circ_{37}^{-1} + 2.77 \times 10^{-3}$ for non-self complementary strands, it is possible to estimate $\Delta G^\circ_{37}$ from $T_m$ values—the most easily available experimental parameter. Also, this equation agrees well with thermodynamic data measured in 100 mM NaCl reported by Lesnik and Freier (19,20) (data not shown). Theoretically, approximation of the straight line between $T \cdot \Delta S^\circ$ and $\Delta H^\circ$, ignoring its intercept, can yield a linear equation between $T_m^{-1}$ and $\Delta G^\circ_{37}^{-1}$; however, the duplexes with a lower stability deviate from linear plots due to the limitation of the approximation. As a result, the equations give good theoretical agreement with the two linear plots in Figure 2B ($T_m^{-1} = -3.43 \times 10^{-3} \cdot \Delta G^\circ_{37}^{-1} + 2.80 \times 10^{-3}$ for self complementary strands and $T_m^{-1} = -3.07 \times 10^{-3} \cdot \Delta G^\circ_{37}^{-1} + 2.80 \times 10^{-3}$ for non-self complementary strands). Moreover, the fact that the relationship between $T_m^{-1}$ and $\Delta G^\circ_{37}$ is derived from thermodynamic equations means the reciprocal correlation of $T_m$ and $\Delta G^\circ_{37}$ could be applied for any oligonucleotide concentration and to duplexes including non-Watson–Crick pairs such as mismatches. Note that both plots in Figure 2A and B were the same regardless of DNA–DNA, RNA–RNA or RNA–DNA duplexes.

Sodium ion concentration sensitivity of duplex stabilities

Effects of sodium ion concentration on $T_m$ for five kinds of DNA–DNA, RNA–RNA and RNA–DNA duplexes with scrambled sequences were measured. Figure 3 shows $T_m$ values for $d(ACCGCA)/d(TGCGGT)$, $d(GCCAGTTAA)/d(TTAACTGGC)$, $r(GCCAGUUAA)/d(TTAACTGGC)$, $r(AUUGGAUACAAA)/r(UUUGUAUCCAAU)$ and $d(AUUGGAUACAAA)/-d(TTTGTATCCAAT)$ measured at several NaCl concentrations. All of these duplex stabilities decreased with decreasing NaCl concentration and indicated a linear dependency of their $T_m$ values on the logarithm of the sodium ion concentration. A good correlation for $T_m$ versus log[Na+] plots in the range of 1 M to 10 mM NaCl for each duplex was observed; however the slopes of these lines were different. Although $d(GCCAGTTAA)/d(TTAACTGGC)$ and $r(GCCAGUUAA)/d(TTAACTGGC)$, and $r(AUUGGAUACAAA)/r(UUUGUAUCCAAU)$ and $r(AUUGGAUACAAA)/d(TTTGTATCCAAT)$ have the same number of phosphate groups, effects of the sodium ion concentration on...
their \( T_m \) values were different. A more stable duplex was enhanced more by increments of sodium ion concentration; 
\[
\frac{d_T}{d \log [Na^+] = m/2.303}
\] 
and \( \Delta \tau \) values were estimated from data previously measured in 1 M NaCl (6) and those in 100 mM NaCl-phosphate buffer (Table 1) as described in the text. These points were fitted to a straight line through the (0,0) point with a least-squares calculation.

**Effects of monovalent and divalent cations on nucleic acid duplex stability**

Considering the above results, we thought it advisable to investigate cation effects on nucleotides using DNA duplexes. Seventeen kinds of cation chlorides, MCl\(_x\) (LiCl, NaCl, KCl, CsCl, MgCl\(_2\), CaCl\(_2\), BaCl\(_2\), MnCl\(_2\), NiCl\(_2\), CuCl\(_2\), ZnCl\(_2\), SnCl\(_2\), PbCl\(_2\), YCl\(_3\), LaCl\(_3\) and GdCl\(_3\)) were examined for their ability to stabilize the d(GCCAGTTAA)/d(TTAACTGGC) duplex. Cation chlorides of CoCl\(_2\), NiCl\(_2\), ZnCl\(_2\), YCl\(_3\), LaCl\(_3\) and GdCl\(_3\) did not yield a melting curve for the duplex, but obscured its absorbance band around 260 nm. In contrast, CuCl\(_2\), PbCl\(_2\) and SnCl\(_2\) precipitated under the experimental conditions used. Figure 5A shows melting curves of the duplex in 10 mM LiCl, NaCl, KCl, CsCl, MgCl\(_2\), CaCl\(_2\), MnCl\(_2\), CaCl\(_2\), BaCl\(_2\)-cyclohexylamine as expected, monovalent cations enhanced \( T_m \) of the duplex but divalent cations stabilized the duplex more effectively. Linear correlations of the \( T_m \) value with the cation concentration are indicated in Figure 5B, although \( T_m \) values measured in 100 mM divalent cation chlorides deviated from the linear plots. Monovalent cations (Na\(^+\), K\(^+\) and Cs\(^+\)) stabilized the duplex to the same extent over the salt concentration examined here. Li\(^+\) was slightly more effective on the duplex stabilization than Na\(^+\), K\(^+\) and Cs\(^+\) by \(-1.5^\circ C\). This curious Li\(^+\) effect has also been reported for a polynucleotide duplex (33). One possible reason is due to a higher ionic character (Li\(^+\) ion has only s-orbitals) compared with the other monovalent cations (34). For alkaline earth metals, smaller atomic numbers seemed to correlate better with the duplex stabilization. MgCl\(_2\) was the most effective for duplex stabilization. When the same salt concentration was examined, \( T_m \) values of the duplex were in the following order; MgCl\(_2\) > CaCl\(_2\) > MnCl\(_2\) > BaCl\(_2\) > LiCl > NaCl > KCl > CsCl. To achieve the same \( T_m \) values with divalent cation chlorides as that in 100 mM NaCl (29.9°C) requires only 0.7 mM for MgCl\(_2\), 1.6 mM for CaCl\(_2\), 2.8 mM for MnCl\(_2\) and 4.3 mM for BaCl\(_2\). Thus, from 140 to 20 times lower concentrations of these divalent cation chlorides can provide the same duplex stability as 100 mM NaCl. The stabilization effect of magnesium ion on the DNA duplex is about 140 times larger than that of sodium ion, which is similar to the ratio of association constants for a B-DNA duplex with hydrated Na\(^+\) (150 M\(^{-1}\)) and with hydrated Mg\(^{2+}\) (12 800 M\(^{-1}\)) determined by NMR (35). Moreover, sensitivity of \( T_m \) value to the cation concentration (d \( T_m \)/d \log[M\(^{2+}\)]) was similar (8.8 ~ 8.9°C) for Na\(^+\), K\(^+\) and Cs\(^+\) monovalent cations, while those for divalent cations were slightly different, 5.6, 5.4, 5.3 and 5.2°C for Mg\(^{2+}\), Ca\(^{2+}\), Mn\(^{2+}\) and Ba\(^{2+}\), respectively. The difference of d \( T_m \)/d \log[M\(^{2+}\)] between Na\(^+\) and Mg\(^{2+}\) was not expected owing to \( T_m /\Delta H^\circ \) values are not so different according to the thermodynamic parameters in Table 2. Thus, the \( \Delta \tau \) contributions are dominant for the difference of d \( T_m \)/d \log[M\(^{2+}\)] between Na\(^+\) and Mg\(^{2+}\) as suggested from equation 7. Assuming \( \beta \) values for divalent cation chlorides are the same as the \( T_m \) dependency on the Na\(^+\) concentration, because d \( T_m /d \log[Na^+] \) is proportional to \( n/m \) when \( \alpha/\beta \) is constant suggested from equation 7. Therefore, d \( T_m /d \log[Na^+] \) values for these oligonucleotides (in the range of 11.5–5.2°C) less than those reported for polynucleotide duplexes, 17.5°C for poly(d(A)-poly(d(T)), 19.6°C for poly(rA)-poly(rU) (10,28) might be the contribution of the \( n \) term.

**Figure 4.** Plot of (d \( T_m /d \log[Na^+] \))·m/2.303 versus \( –\Delta \tau \) for DNA duplexes listed in Table 1. \( d_T \), \( \Delta \tau \) and \( \Delta H^\circ \) values were estimated from data previously measured in 1 M NaCl (6) and those in 100 mM NaCl-phosphate buffer (Table 1) as described in the text. These points were fitted to a straight line through the (0,0) point with a least-squares calculation.
that for NaCl, $\Delta n$ values were estimated from equation 7 as well as those from equation 6 shown in Table 3. Li$^+$, Na$^+$, K$^+$ and Cs$^+$ showed the same $\Delta n$ values within the experimental error (1.33 ± 0.02), but divalent cations were remarkably different from each other (0.70 ± 0.01), and the order is Mg$^{2+}$ > Ca$^{2+}$ > Mn$^{2+}$ > Ba$^{2+}$, which is the same as their ability to stabilize the duplex. Also, $\Delta n$ values for Na$^+$ and Mg$^{2+}$ determined by $T_m$ versus log $[M^{n+}]$ plots are similar to those obtained from log $K_a$ versus log $[M^{n+}]$ plots. More than half of $\Delta n$ anticipated for divalent cations and the different $\Delta n$ requirements among divalent cations disagree with their valence, meaning not only the electronegativity of nucleotides decides the number of these cations bound to the duplex, but also the electronegativity of cations. Thus, the affinity of cations with duplexes would affect the $\Delta n$ values and duplex stability.

CD spectra of d(GCCAGTTAA)/d(TTAACTGGC) in NaCl and in MgCl$_2$ show lesser cation effects on the global conformation of its duplex (data not shown) but did influence the duplex stability as shown in Table 2. A buffer including both NaCl and MgCl$_2$, since it is often used in order to stabilize the secondary structure of nucleotides (36) and to allow ribozyme activity (37–40), was also employed. These cations are considered to have different binding sites on nucleotides; Na$^+$ binds more with the phosphate group and Mg$^{2+}$ prefers the N7 of purine bases (41). Figure 6 indicates $T_m$ values for d(GCCAGTTAA)/d(TTAACTGGC) measured in various MgCl$_2$ concentrations in the presence or absence of 100 mM NaCl. From the viewpoint of an ionic strength, MgCl$_2$ with 100 mM NaCl is more effective for the duplex stabilization than MgCl$_2$ alone. However, $T_m$ values measured in >1 mM MgCl$_2$ concentration with 100 mM NaCl were similar to those in MgCl$_2$ alone, and $T_m$ values in <1 mM MgCl$_2$ concentration with 100 mM NaCl were similar to these in 100 mM NaCl alone (29.9°C). This was also observed from the thermodynamic values in Table 2. Williams et al. (15) reported $T_m$ values measured in several Mg$^{2+}$ concentrations in the presence of Na$^+$ and showed similar $T_m$ dependencies on Mg$^{2+}$ concentration as shown in Figure 6. Thus, establishment of the prediction system for duplex stability under MgCl$_2$ with NaCl might be easily possible. Furthermore, these results suggest that Na$^+$ and Mg$^{2+}$ compete with each

table 2. Thermodynamic parameters of d(GCCAGTTAA)/d(TTAACTGGC) formation in NaCl and/or MgCl$_2$ containing buffer

<table>
<thead>
<tr>
<th>Salt concentration</th>
<th>$T_m$ vs log $C_1$ parameter</th>
<th>curve fit parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\Delta H$ $\Delta S$ $\Delta G^{\circ}$</td>
<td>$T_m$ $\Delta H$ $\Delta S$ $\Delta G^{\circ}$ $T_m$</td>
</tr>
<tr>
<td>/mM</td>
<td>/kcal mol$^{-1}$ /cal mol$^{-1}$ K$^{-1}$ /kcal mol$^{-1}$</td>
<td>/°C /kcal mol$^{-1}$ /cal mol$^{-1}$ K$^{-1}$ /kcal mol$^{-1}$ /°C</td>
</tr>
<tr>
<td>NaCl</td>
<td>400 -69.6±2.6 -195.7±7.6 -8.04±0.33 36.8 -72.7±3.0 -208.5±9.6 -8.01±0.12 36.7</td>
<td>100 -70.5±1.8 -205.9±3.2 -6.93±0.16 32.0 -71.5±4.0 -208.2±12.8 -6.89±0.16 31.9</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>100 -63.4±1.6 -177.1±4.6 -8.68±0.19 39.0 -64.3±8.7 -176.7±27.0 -8.63±0.31 39.7</td>
<td>10 -67.4±2.1 -192.6±6.2 -7.67±0.17 36.0 -64.5±2.8 -183.3±8.9 -7.63±0.10 34.8</td>
</tr>
<tr>
<td></td>
<td>1 -63.2±2.4 -182.4±7.3 -6.59±0.31 29.8 -60.1±2.5 -172.7±8.3 -6.54±0.09 29.2</td>
<td>100 -69.6±2.4 -197.4±7.0 -8.43±0.30 38.5 -65.6±2.6 -184.2±8.0 -8.45±0.21 38.7</td>
</tr>
<tr>
<td></td>
<td>1 -63.8±2.3 -190.7±6.8 -7.60±0.29 35.1 -64.7±2.4 -193.6±7.5 -7.79±0.19 35.6</td>
<td>1 -63.6±4.7 -182.5±13.7 -7.01±0.73 31.8 -61.7±4.1 -176.2±13.3 -7.09±0.27 32.1</td>
</tr>
</tbody>
</table>

* All experiments were conducted in buffer containing NaCl/10 mM Na$_2$PO$_4$/1 mM Na$_2$EDTA or MgCl$_2$/10 mM sodium cacodylate (pH 7.0).

* Melting temperatures were calculated at the total oligomer strand concentration of 8 µM.

Table 3. The number of released cations when a duplex forms determined by two methods

<table>
<thead>
<tr>
<th>Cation (M$^{n+}$)</th>
<th>$\Delta n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Li$^+$</td>
<td>1.35 ± 0.01</td>
</tr>
<tr>
<td>Na$^+$</td>
<td>1.33 ± 0.02</td>
</tr>
<tr>
<td>K$^+$</td>
<td>1.33 ± 0.01</td>
</tr>
<tr>
<td>Cs$^+$</td>
<td>1.35 ± 0.04</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>0.76 ± 0.02</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>0.74 ± 0.01</td>
</tr>
<tr>
<td>Ba$^{2+}$</td>
<td>0.70 ± 0.02</td>
</tr>
<tr>
<td>Mn$^{2+}$</td>
<td>0.72 ± 0.02</td>
</tr>
</tbody>
</table>

$\Delta n$ values are determined from the equations 6 and 7.

* $\alpha$ and $\beta$ values for monovalent cations are used 0.92 and 58.1, respectively. Those for divalent cations are 0.88 and 58.1, respectively.

Insights of cation binding to nucleic acid duplexes

Both stacking interactions and hydrogen bonds are widely understood to stabilize nucleic acid duplex structure (41,42). Here, we have revealed the importance of $\Delta n$ values on the duplex stability. The importance of cation binding for duplex structure are also reported. For instance, magnesium and
potassium ions are needed for the folding of the *Tetrahymena* group I intron P4-P6 domain (37,38). Also, for d(CGCGAATTCGCG)₂, Na⁺ and K⁺ ions partially occupy the primary water spine located at the minor groove of the duplex, and the bent structure at the AT–GC junction is due to the bound sodium ion bridging both nucleotide strands (43,44). Considering these facts, it is probable that cations bound to a double helix passively affect nucleic acid duplex stability (39,45).

It has been considered that cations, especially alkali metal ions, bind only to phosphate groups of nucleic acids (41). However, recent studies concerning cations and nucleotides arising from *ab initio*, semi-empirical and empirical results reveal other possibilities. Computational *ab initio* calculations often indicate N7 and O6 of guanine as the preferential cation binding sites. N7 of adenine is also a probable binding site (33,46). Longtime computer simulations to a nanosecond with AMBER 4.1 revealed the number of hits of sodium ions with A-RNA and B-DNA most frequent for GpG step at N7 and O6 of guanine bases (47). Moreover, hydrated magnesium ions are found in the major groove located between G/C base pairs of d(CGCGAATTGC)₃, by high resolution structural analysis (43). These results support preferential cation binding sites of N7 and O6 of guanine bases as well as phosphate groups, which are components of one of the most stable nearest-neighbor base pairs of CG/CG (6–9,24). Here, we have demonstrated that the number of Na⁺ required for a duplex formation was related to the duplex stability. The duplex stability is accounted for by nearest-neighbor interactions (4–9). Accordingly, it is possible that newly bound Na⁺ when a duplex forms binds with nucleobases as well as phosphate groups, though its affinity toward nucleobases, perhaps N7 and O6 of a guanine base, is much lower than that of Mg²⁺. It is probable that naked or hydrated cations required for duplex formation are preferentially coordinated by both phosphate groups and nucleobases. The influence of nucleobases on the salt effect suggests that there are preferential base pairs or nearest-neighbor base pairs for the counter cation binding. Such sequence-dependent bindings might affect all nearest-neighbor parameters. As mentioned, it was impossible to distinguish among duplex species on the basis of linear plots of the thermodynamic values for duplex formation. This finding means that 2'-OH of RNA strand, sugar puckering and the methyl group of thymine may affect sequence-dependent cation bindings. Theoretical studies of cation distribution around a DNA duplex were performed based on counterion condensation (CC), Monte Carlo (MC) or Poisson-Boltzmann (PB) methods (46). These calculations take into account electronegative points located only at the phosphates. However, if cations interact with nucleobases as well as the phosphates, it will be necessary to reconsider this model by including the possible cation binding sites in both grooves of the duplex surface. Such a consideration might reduce the end-effects on cation distributions of a duplex (48).
Johnson et al. (49) reported that differences in the stacking energies under high ionic conditions are conserved even at a lower salt condition. This concept means the base pair geometry is not significantly affected by the salt condition. Our own report would be consistent with the results obtained here in that duplex stabilities in 1 M NaCl are linearly correlated with lower salt condition. This concept means the base pair geometry is conserved even at a high ionic condition, which explains the salt effect as an oligomer length dependence.

ACKNOWLEDGEMENTS

We thank Dr R. I. Gunport for his comments on the manuscript. This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports, and Culture, Japan, and Grants from Research for the Future Program of the Japan Society for the Promotion of Science.

REFERENCES