Capture of cationic ligands bound diffusely to base pairs during DNA refolding†

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We obtained the intrinsic binding affinity for metal ions, polyamines, and oligolysine peptides diffusely bound to base-paired sites in DNA by monitoring the shift of the hairpin–duplex equilibrium of the self-complementary DNA sequences, which can be widely used for capturing cationic ligands bound diffusely to nucleotide base pairs.

Nucleotide folding is accompanied by the association of cationic molecules that shield the electronegative potential of nucleotide phosphates in order to bring them into close proximity in space. The majority of such cations nonspecifically bind to the phosphate groups through Coulombic interactions in the condensation layer, while the binding to defined nucleotide sites often occurs in the tertiary folding and they may have catalytic roles.[1] Metal ions in the condensation layer predominantly associate diffusely with base-paired nucleotides and exchange rapidly with bulk ions, in which the charge neutralization is generally more effective as a result of higher cation valence. Polyamines, such as spermidine and spermine, and basic amino acid residues in proteins also contribute to the nucleotide charge neutralization. Evaluations of the binding affinity of these cationic ligands to nucleotides are important for understanding the energetic contribution of the cation binding to nucleotide folding events. Although cations bound at defined sites have been well studied, providing, *e.g.*, the association constant for Mg^{2+} bound with tRNAs typically on the order of 10^5 M or greater in the presence of a low amount of monovalent cation (10 mM or less),[2] the binding parameters for the diffusely bound cations, which usually have a weaker binding affinity than those associating at specific sites, have not been thoroughly studied due to energetic coupling with the nucleotide folding[3] and the limited availability of experimental systems to capture the diffusely bound cations.

Generally, cations stabilize the folded conformations of nucleotides. According to the polyelectrolyte theory, stabilization to a bimolecular duplex can be more effective than to a hairpin structure of the same nucleotide length due to fewer inter-phosphate repulsions in the loop nucleotides.[4] This property in nucleotides can lead to a shift in the hairpin–duplex equilibrium of a self-complementary sequence toward the bimolecular duplex by elevating the ionic strength.[5] In this study, we explored the self-complementary DNA sequences showing the structural transition from a hairpin to a bimolecular duplex upon adding cationic ligands, and evaluated the intrinsic binding affinity of the diffusely bound cations by monitoring the DNA refolding reaction.

We tested several self-complementary DNA oligomer sequences, and it was found from native PAGE (polyacrylamide gel electrophoresis) experiments[6] that the 14-mer sequence containing the C/G base pairs in the middle of the sequence (**d14cg**, 5′-GCAAGCCGGCTTGC-3′) exhibited a hairpin structure at 10 mM NaCl, but formed a fully matched bimolecular duplex at 1 M NaCl and 37 °C (Fig. S1†). The structural transition on elevating the sodium ion concentration resulted primarily from an increase in the cation condensation around the central CG nucleotides, being the loop nucleotides at 10 mM NaCl and forming Watson–Crick base pairs at 1 M NaCl (Fig. 1A).[5d] The PAGE experiments revealed an increase in the bimolecular duplex fraction of **d14cg** with the increase in the NaCl concentration in a two-state fashion (Fig. 1B), and the NaCl concentration required

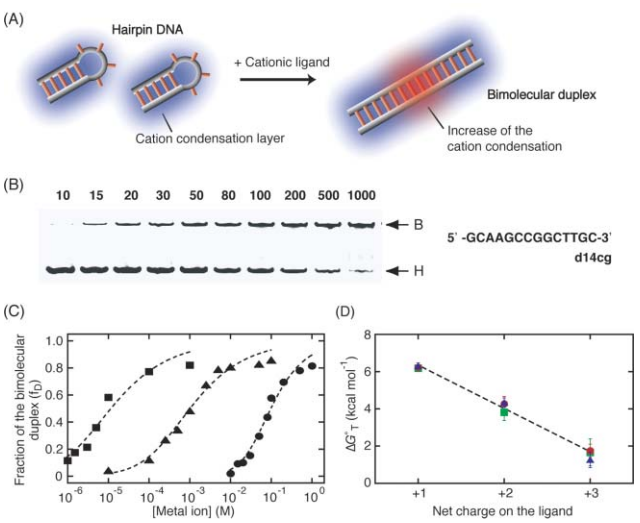

Fig. 1 (A) Illustration of the structural transition of a self-complementary DNA by the association of cationic ligands. (B) The changes in the molar fractions of the hairpin form (indicated by H) and the bimolecular duplex (indicated by B) of **d14cg** by changing the NaCl concentration (mM) indicated at the top of the picture. (C) Dependence of the fraction of the bimolecular duplex of **d14cg** on the NaCl (circles), $MgCl_2$ (triangles), and $[Co(NH_3)_6]Cl_3$ (squares) concentrations. The broken lines represent the curve fittings. (D) Plots of $\Delta G°_T$ *versus* the net charge of the series of metal ions (red), polyamines (blue), and oligolysine peptides (green).

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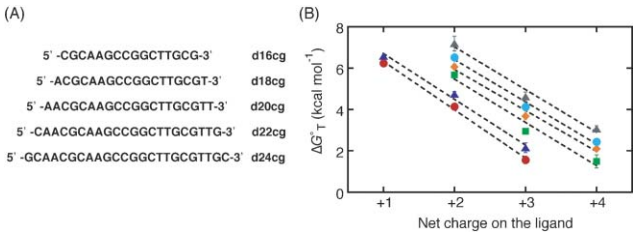

Fig. 2 (A) Oligonucleotide sequences of **d16cg**, **d18cg**, **d20cg**, **d22cg**, and **d24cg**, fluorescently labeled with 6-FAM at their 5′-termini. (B) Plots of ΔG°_{T} *versus* the net charge on the cationic ligands for **d14cg** (red), **d16cg** (blue), **d18cg** (green), **d20cg** (orange), **d22cg** (cyan), and **d24cg** (gray). Each ΔG°_{T} value was averaged among the different series of cationic molecules and the error bar indicates the standard deviation for the cations with the same net charge.

for forming the bimolecular duplex at a 0.5 molar fraction, $[NaCl]_{0.5}$, was 100 mM (Fig. 1C). The equilibrium shift to the bimolecular duplex was also observed when other cations in the series of metal ions (Mg^{2+} and $[Co(NH_3)_6]^{3+}$ as well as Na^+), polyamines (NH_4^+ as the charged group of polyamines, putrescine, spermidine, and spermine), and the oligolysine peptides (Lys monomer, Lys_2, Lys_3, and Lys_4 with free N and C termini) were used under the 10 mM NaCl background at 37 °C.[7] Lower cation concentration with a higher net charge was required for the DNA structural transition (Figs. 1C and S2†), and the $[L]_{0.5}$ values, the cationic ligand concentration for adopting the bimolecular duplex at a 0.5 molar fraction, were similar among the cations with the same net charge, *e.g.*, the values of $[L]_{0.5}$ for **d14cg** with the trivalent cations of $[Co(NH_3)_6]^{3+}$, spermidine, and Lys_3 were 12 ± 2, 9.3 ± 0.7, and 7.3 ± 1.1 μM with a binding cooperativity parameter (n) close to unity (0.97 ± 0.05, 1.0 ± 0.1, and 0.94 ± 0.09, respectively).[8] The free energy change ΔG°_{T} at 37 °C, describing the overall reaction of the DNA structural transition by cation binding, was calculated using the equation $\Delta G^{\circ}_{T} = RT\ln K_T$ $(= nRT\ln[L]_{0.5} + RT\ln C_t)$, in which R is the gas constant and T is the temperature. Linear correlation plots of ΔG°_{T} *versus* the net charge on the cationic ligand were observed regardless of the series of metal ions, polyamines, and oligolysine peptides (Fig. 1D), consistent with diffuse nonspecific electrostatic interactions with the DNA.

To reveal the influences of the DNA refolding energy on the cation binding parameters, the fully self-complementary DNA sequences of different lengths ranging from 16-mer to 24-mer containing the central CG sequence (**d16cg**, **d18cg**, **d20cg**, **d22cg**, and **d24cg**, shown in Fig. 2A) were further examined.[9] The structural transition was also observed for these DNAs, and the $[L]_{0.5}$ value increased for the longer nucleotides (Table 1). However, the $[L]_{0.5}$ values for each sequence were similar among the cations with the same net charge and their n values were close to unity (Table S1†), as observed for **d14cg**. More importantly, the linear plots of ΔG°_{T} for each DNA *versus* the net charge of the cation (m) showed a slope of −2.1 kcal mol^{-1} ($\Delta G^{\circ}_{charge}$) (Fig. 2B), and the intercept differed among the DNA sequences responsible for the intrinsic DNA refolding energy ($\Delta G^{\circ}_{refold}$). The observation suggests that ΔG°_{T} can be represented by the sum of the intrinsic binding constant of cation with the net charge of m ($m\Delta G^{\circ}_{charge}$) and $\Delta G^{\circ}_{refold}$, and $\Delta G^{\circ}_{charge}$ is energetically decoupled from the nucleotide folding. According to the free energy bonus of 2.1 kcal mol^{-1} (corresponding to a 31-times greater binding affinity at 37 °C) per single net positive charge on the cationic ligands, the intrinsic binding constants for the cations with the net charge of +1, +2, +3, and +4 ($m\Delta G^{\circ}_{charge}$) at 10 mM NaCl and 37 °C were calculated to be 31, 0.98 × 10^3, 3.0 × 10^4, and 0.95 × 10^6 M^{-1}, respectively, despite the DNA sequence. These estimations are in good agreement with previous reports using polymer nucleotides or tRNAs in which the cation binding can be regarded as independent of the nucleotide folding, therefore providing the intrinsic cation binding parameter. The apparent association constant obtained in a solution containing low NaCl concentrations (*ca.* 10 mM) at 25 °C to 20 °C for the nonspecifically bound Mg^{2+} is on the order of 10^3,[2,10] that for the putrescine binding is on the order of 10^3 M^{-1} and the value increases by about 30 times with the increasing number of amino groups on the polyamine;[11] those for the basic oligopeptides with the net charge of +2, +3, and +4 are on the order of 10^3, 10^4, and 10^6 M^{-1}, respectively.[12] Accordingly, it is concluded that our analyses using PAGE provide the intrinsic cation binding parameters which are energetically uncoupled with the nucleotide folding. Notably, although the cations shifting the hairpin–duplex equilibrium are supposedly bound near the CG nucleotides (Fig. 1A), comparisons with the previous results using polymer nucleotides and tRNAs suggest an insignificant influence of the nucleotide sequence on the cation binding affinity.

In summary, we evaluated the binding affinities of metal ions, polyamines, and oligolysine peptides diffusely bound to DNA base pairs by monitoring how cationic ligands shifted the hairpin–duplex equilibrium of the self-complementary DNA

Table 1 The values of ΔG°_{T} (kcal mol^{-1}) at 37 °C for the cationic ligand binding to the DNAs[a]

Ligand[b]	d14cg	d16cg	d18cg	d20cg	d22cg	d24cg
Na^+ (+1)	6.2 ± 0.2	6.7 ± 0.1	7.1 ± 0.1	nd	nd	nd
NH_4^+ (+1)	6.3 ± 0.2	6.6 ± 0.1	6.9 ± 0.1	nd	nd	nd
Lys (+1)	6.2 ± 0.1	6.3 ± 0.2	6.6 ± 0.3	nd	nd	nd
Mg^{2+} (+2)	4.3 ± 0.4	4.8 ± 0.3	5.4 ± 0.2	6.0 ± 0.3	6.6 ± 0.2	7.2 ± 0.1
Putrescine (+2)	4.3 ± 0.3	4.8 ± 0.5	6.2 ± 0.3	6.2 ± 0.3	6.5 ± 0.2	7.2 ± 0.2
Lys_2 (+2)	3.8 ± 0.5	4.6 ± 0.3	5.4 ± 0.3	6.0 ± 0.3	6.5 ± 0.3	7.1 ± 0.2
$Co(NH_6)^{3+}$ (+3)	1.8 ± 0.4	2.5 ± 0.3	2.9 ± 0.5	3.9 ± 0.5	4.1 ± 0.1	4.4 ± 0.1
Spermidine (+3)	1.3 ± 0.4	1.5 ± 0.5	2.9 ± 0.4	3.6 ± 0.4	4.0 ± 0.6	4.4 ± 0.3
Lys_3 (+3)	1.7 ± 0.7	2.5 ± 0.8	3.1 ± 0.7	3.6 ± 0.4	4.3 ± 0.3	5.1 ± 0.3
Spermine (+4)	nd	nd	1.4 ± 0.4	1.9 ± 0.5	2.4 ± 0.3	3.0 ± 0.6
Lys_4 (+4)	nd	nd	1.6 ± 0.8	2.3 ± 1.0	2.5 ± 0.8	3.2 ± 0.3

[a] nd indicates that the parameter could not be determined due to an affinity too high or too low to obtain a reliable value. [b] The value in parentheses indicates the net charge of the ligand.[7]

oligonucleotides. Although the number of cations bound with nucleotides usually differs depending on the valence and the chemical structure of the cation, our approach provides the intrinsic binding affinity with a lower binding cooperativity. This could have resulted from the restriction in the binding site near the central CG nucleotides because what we measured by this approach was only the cations required for the structural transition. Because the PAGE experiments are simple and quite useful, the nucleotide refolding study can be widely used for capturing diffusely bound cations even when they are bound very weakly, as observed for monovalent cations. In addition, it also enables the measurement of cations bound to other structures formed by short nucleotides. Such binding parameters are also useful for theoretical calculations and computational simulations involving nucleotide folding and cation binding.

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6 The samples were prepared as reported previously.[5d] After annealing at 90 °C for 5 min of a 1 μM DNA strand fluorescently-labeled with 6-FAM (6-carboxyfluorescein) at its 5′-terminus in the buffer (25 mM HEPES at pH 7.0) containing the appropriate cationic ligand in addition to 10 mM NaCl and 0.1 mM Na_2EDTA, the solution was cooled down to 37 °C at the rate of -1 °C min^{-1}, followed by loading on the native 20% polyacrylamide gel in a room at 37 °C. The fraction of bimolecular duplex ($f_D = B_D/(B_H + B_D)$) was evaluated from the emission intensities of the bimolecular duplex (B_D) and the hairpin (B_H). Our previous study confirmed that the $[L]_{0.5}$ determined by native PAGE was the same as that obtained by the change in the CD spectra.[5d] It is noted that the interconversion of the hairpin and the bimolecular duplex and their dissociations were slow under these conditions, although the shorter DNAs of 12-mer and 10-mer sequences were dissociated into a single strand during the PAGE (Fig. S1†).

7 The lowest pK_a of spermine and spermidine is close to 8.2, and the pK_a can be shifted upward by the electronegative density in the proximity of the nucleotide phosphates. The data of the exchange-inert $[Co(NH_3)_6]^{3+}$ suggest outer-sphere binding. In addition, less influence from the charges at the amino and carboxyl termini of the oligolysine peptides (purchased from Sigma) on the binding parameters was confirmed by comparing the data using the oligolysine capped with an acetyl group and an amino group at the amino and carboxyl termini, respectively (data not shown). The CD spectra indicated fewer perturbations of the bimolecular duplex conformation by the polyamines that may bind in the DNA grooves or delocalize along the DNA [H. Deng, V. A. Bloomfield, J. M. Benevides and G. J. Thomas, *Nucleic Acids Res.*, 2000, **28**, 3379; A. A. Ouameur and H.-A. Tajmir-Riahi, *J. Biol. Chem.*, 2004, **279**, 42041], while being slightly changed when the Lys monomer and the oligolysine peptides were examined (Fig. S3†).

8 The values of $[L]_{0.5}$ were calculated by the curve fitting of the Fig. 1B plots to the equation $f_D = \{4[L]_0^n + K_T/C_t - (8[L]_0^n K_T/C_t + K_T^2/C_t^2)^{0.5}\}/4[L]_0^n\}$, where f_D is the fraction of the bimolecular duplex, n is the binding cooperativity parameter, K_T is the thermodynamic equilibrium constant ($K_T = [H]^2[L]^n/[D] = C_t[L]^n_{0.5}$ or $RT\ln K_T = nRT\ln[L]_{0.5} + RT\ln C_t$) for the reaction, $2H + nL \rightleftharpoons D$ (H, L, and D indicate the hairpin, the cationic ligand, and the bimolecular duplex, respectively), by assuming the cationic ligand concentration, $[L]_0$ was in excess of the total DNA strand concentration, C_t. This assumption seemed to be invalid for the DNAs exhibiting a structural transition with low cation concentrations (*e.g.*, **d14cg** with tetravalent cations of spermine and Lys_4), thus they were omitted in the Figs. 1D and 2B plots. The n values obtained here were close to 1.0 and no greater than 1.6 (Table S1†). The K_T and n values were also calculated from the observed equilibriums ($K_{obs} = [H]^2/[D]$) at the given cation concentrations ($K_T = K_{obs}[L]^n$), which provided parameters similar to those obtained by the Fig. 1C plots.

9 The corresponding DNAs containing central AT sequences showed a lower structural transition ability even on adding 1 mM spermine, probably due to the reduced stability of the bimolecular duplex compared with those containing the central CG sequence (Fig. S4†).

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