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Thermodynamic Investigation of the Formation of Complexes between Norfloxacin and Various Mononucleotides

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The fluorescence of norfloxacin was quenched by various nucleotides. The ratio of the fluorescence intensities in the absence and presence of nucleotide was linearly dependent on nucleotide concentration, suggesting that quenching occurred through the formation of nonfluorescent norfloxacin-nucleotide complexes. The gradient of the linear relationship represented the equilibrium constant of complex formation; it decreased with increasing temperature. The slopes of van't Hoff plots constructed from the temperature-dependent equilibrium constants were positive in all cases, indicating that complex formation was energetically favorable – i.e., exothermic, with negative Gibb's free energy. The equilibrium constant increased when triphosphate was used instead of monophosphate. It also increased when the oxygen at the C'_2 position of the nucleotide was removed. Both enhancements were due to entropic effects: entropy decreased when complexes with AMP or GMP formed, while it increased when norfloxacin complexed with ATP, GTP, dAMP and dGMP.

Key Words : Nucleotides, Norfloxacin, Enthalpy, Entropy, Gibbs free energy

Introduction

Quinolones are clinically important antibacterial agents and their development represents a major advance in antimicrobial chemotherapy. They inhibit the action of bacterial type II topoisomerase, which unwinds supercoiled DNA prior to replication and transcription, thereby affecting the efficiencies of those processes.[1] The biological and medicinal importance of quinolone antibiotics has led to numerous studies of their interactions with DNA. However, the binding target of quinolones in living cells has not yet been identified: i.e., whether they bind with supercoiled DNA, gyrase, or DNA-gyrase complexes. Norfloxacin (Figure 1), a representative quinolone, has been reported to bind to DNA or DNA-gyrase complexes but not to protein.[2,3] Subsequent work has revealed that norfloxacin binds preferentially to single-stranded DNA, with only weak binding to double-stranded DNA.[4,5] When gyrase and nonhydrolyzable ATP analogue were added to relaxed circular DNA, a specific single-stranded DNA pocket was induced by gyrase and four norfloxacins were proposed to bind cooperatively at this pocket.[6] In the pocket, norfloxacin was stabilized by various forces, including the formation of hydrogen bonds, π-π interactions between norfloxacin rings, and tail-to-tail hydrophobic interactions. A different binding mode of norfloxacin to super coiled DNA was proposed in the presence of Mg^{2+} ions:[7,8] the ions could act as bridges between DNA phosphate groups and carbonyl and carboxyl moieties of the norfloxacin.

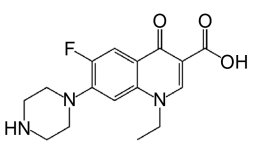

Figure 1. Norfloxacin.

Quinolones have also been reported to bind to native and synthetic DNA in the absence of cofactors such as gyrase, ATP analogues and Mg^{2+} ions. Although electrophoresis experiments have shown that norfloxacin is able to unwind the DNA double helix[9] and a linear dichroism study has demonstrated that the molecular plane of norfloxacin is near parallel to the DNA base-pair plane,[10] intercalative binding is unlikely because the unwinding angle of norfloxacin is much smaller than those of classical intercalators such as ethidium.[9,11] A detailed linear dichroism study showed that the angle between the norfloxacin molecular plane and the DNA helix axis was 67°-86°, within the range of the base inclination angle of B-form DNA, thus not favoring intercalative binding.[11] Subsequent studies using various spectroscopic and theoretical methods by the same group[11-17] have shown that quinolones preferentially bind to single-stranded DNA over double-stranded DNA. They also preferred GC sequences. The amine groups of guanine bases protruding from the minor groove were responsible for this preference of GC over AT base pairs. The interaction of ofloxacin, which possesses a chiral carbon on the oxazine ring, with DNA is interesting to note. The binding affinity of its *S* enantiomer, has been reported to be 5 × that of its *R*-enantiomer.[18] The antibiotic activity and the inhibition of topoisomerase II by the *S* enantiomer has been reported to be much greater than by the *R* enantiomer.[19-21] This highlights

the importance of binding mode and affinity to the biological activities of quinolones.

The debate surrounding the proposed binding modes indicates that the forces governing the binding of quinolones to DNA are still not fully understood. This needs to be resolved to facilitate the rational design of this class of drug. This work reports thermodynamic aspects of adduct formation between quinolone and various nucleotides. Norfloxacin was used as a representative quinolone. Nucleotides contained either mono- or tri- phosphate groups. 2'-Deoxy nucleotides were also tested. Tested nucleotides were guanine, adenine and inosine because the drug's interactions with pyrimidine bases are known to be weak.

Materials and Methods

Materials. All chemical were obtained from Sigma-Alderich and used without further purification. The concentrations of norfloxacin and the nucleotides were measured spectrophotometrically using the extinction coefficients: ε_{273nm} = 37500 $M^{-1}cm^{-1}$ for norfloxacin, ε_{253nm} = 13700 $M^{-1}cm^{-1}$ for GMP (= GTP = 2'deoxyGTP), ε_{259nm} = 15400 $M^{-1}cm^{-1}$ for AMP (= ATP = 2'deoxyATP), and, ε_{248nm} = 13700 $M^{-1}cm^{-1}$ for ITP. All experiments were performed in 5 mM cacodylate buffer at pH 7.0. Absorption spectra were recorded on a Cary 500 spectrophotometer.

Modeling Study. Since crystal structures of norfloxacin were not present, it was optimized by Hartree-Fock using a 6-31G* basis set in the Gaussian 03 program package. The nucleotides' structures were obtained from the database of the Hyperchem 7.5 program. Molecular modeling was performed using the Hyperchem package.

Measurement. The fluorescence of norfloxacin is quenched upon addition of DNA or nucleotide. When quantum yield is negligible in the presence of quencher compared with in the absence of quencher, the fluorescence intensities in the presence (F) and absence (F_0) of quencher can be expressed as Stern-Volmer plots according to equation (1).[22]

$$\frac{F_0}{F} = 1 + K[Q] \tag{1}$$

Where K is the Stern-Volmer quenching constant and $[Q]$ the concentration of quencher, i.e. nucleotide. If the Stern-Volmer plot shows a linear relationship, the fluorescence quenching follows either a dynamic or static mechanism. During dynamic quenching, the energy of the excited fluorophore transfers upon collision with the quencher. During static quenching, the ground-state fluorophores form non-fluorescent complexes with quenchers. Therefore, the Stern-Volmer quenching constant can be considered the equilibrium constant for fluorophore-quencher complexation. The efficiency of dynamic quenching increases with increasing temperature because more collisions result from the molecules' increased speeds. Increasing temperature decreases the efficiency of static quenching because the complexes are usually unstable at higher temperatures – this was observed in this study.

The log of the measured equilibrium constant can be plotted with respect to the inverse of absolute temperature to construct a van't Hoff plot (equation (2)). Gibb's free energy can also be obtained from basic thermodynamics (equation (3)).

$$\ln K = \left(-\frac{\Delta H^o}{RT}\right) + \frac{\Delta S^o}{R} \tag{2}$$

$$\Delta G^o = -RT \ln K \tag{3}$$

The slope and the y-intercept of the van't Hoff plot give the enthalpy and entropy changes of complex formation. Fluorescence spectra were recorded on a Jasco FP-777 fluorimeter. Slit widths for excitation and emission were both 5 nm. Temperature was controlled by a water bath.

Results

Fluorescence Quenching by AMP and GMP. Aqueous norfloxacin fluoresced strongly, with maximum emission at 418 nm (Figure 2). The addition of any DNA, e.g. single- or double-stranded DNA, and supercoiled DNA, various synthetic polynucleotides, and mono-nucleotides quenches this fluorescence. 2 mM GMP significantly reduced the fluorescence intensity (Figure 2), suggesting that the fluorescence of the norfloxacin-GMP complex was negligible. Some fluorescence was observable in the presence of GMP, it was due to remaining unquenched norfloxacin. Therefore the construction of simple Stern-Volmer plots was justifiable. Stern-Volmer plots for the fluorescence quenching of norfloxacin by AMP and GMP (Figure 3(a)) were linear, indicating that the quenching mechanism was either static or dynamic. Their similar Stern-Volmer slopes show that the quenching efficiencies of AMP and GMP were comparable. This appears in contrast with the reported quenching efficiencies of poly[d(G-C)$_2$] and poly(dG)·poly(dC) being much greater than those of poly[d(A-T)$_2$] and poly(dA)·poly(dT).[13] For static quenching, observed here (see below), the slope can be considered the equilibrium constant of non-fluorescent norfloxacin-nucleotide monophosphate complex for-

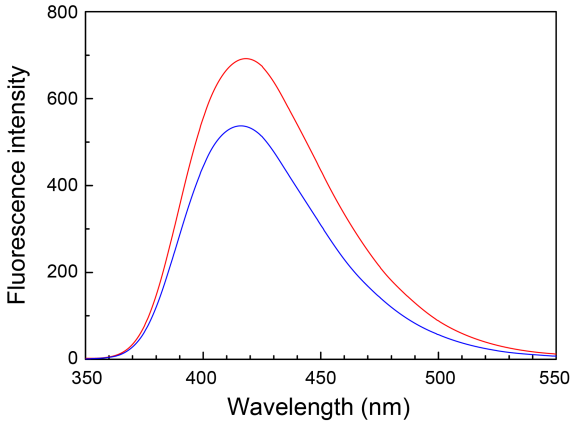

Figure 2. Fluorescence emission spectra of norfloxacin in the presence (blue) and absence (red) of GMP. Norfloxacin was excited at 323 nm. [Norfloxacin] = 1 μM, and [GMP] = 2 mM.

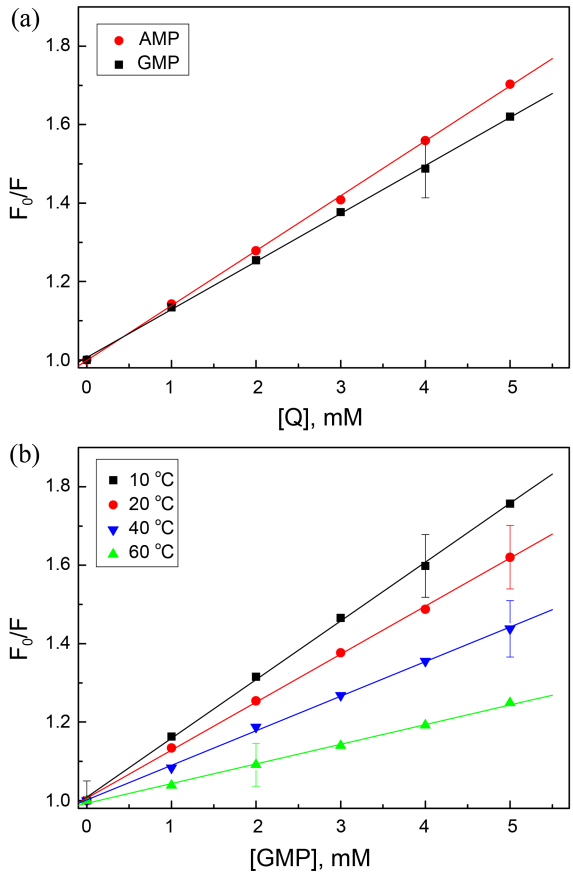

Figure 3. (a) Stern-Volmer plots of norfloxacin fluorescence quenching at 20 °C by AMP (black squares) and GMP (red circles). (b) Temperature-dependent fluorescence quenching by GMP. Representative error bars, denoting standard deviations of three measurements, are shown. Fluorescence intensities were measured through 323 nm excitation and 415 nm emission. Slit widths for both excitation and emission were 5 nm. [Norfloxacin] = 1 μM.

mation. Equilibrium constants of 140 M^{-1} and 122 M^{-1} were found for AMP and GMP at 293 K, respectively, comparable to reported values measured at room temperature.[13] The quenching efficiencies of GMP (Figure 3(b)) and of AMP (data not shown) decreased with increasing temperature. The decrease of slope with increasing temperature supported the formation of nonfluorescent norfloxacin-GMP or -AMP complexes as being responsible for quenching. This is because higher temperatures would lead to more frequent collisions between excited fluorophores and quenchers during dynamic quenching. Figure 4 shows a van't Hoff plot for the temperature-dependent behavior (equation (3)) of norfloxacin-GMP complexation. AMP behaved similarly (data not shown). Both the slope and y-intercept of the van't Hoff plot are positive, indicating that complex formation was exothermic but entropically unfavorable. The calculated changes of entropy and enthalpy, and the equilibrium constants were similar for AMP and GMP (Table 1). Their free energy changes were both negative and of similar magnitude, suggesting that complexation was spontaneous for both AMP and GMP. This shows that the different nucleo-base structures did not affect complexation with nucleotide mono-

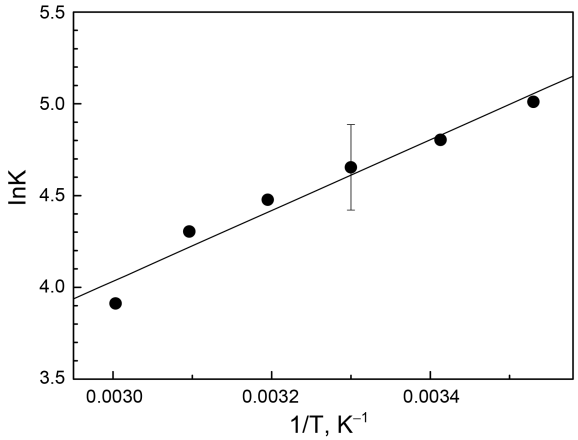

Figure 4. Van't Hoff plot for the association of GMP and norfloxacin constructed from the data in Figure 3(b). AMP behaved similarly.

Table 1. Thermodynamic parameters and equilibrium constants of norfloxacin-nucleotide complexation

Nucleotide	K, M^{-1}	$\Delta H°$, kJ·mol^{-1}	$\Delta S°$, J·mol^{-1}·K^{-1}	$\Delta G°$, kJ·mol^{-1}
AMP	140	-17.23	-17.97	-12.04
GMP	122	-16.03	-14.55	-11.70
ATP	238	-12.03	4.52	-13.33
GTP	501	-14.86	0.74	-15.24
ITP	228	-9.91	11.19	-13.23
2'deoxy AMP	64	-2.95	24.50	-10.13
2'deoxy GMP	247	-12.36	3.58	-13.42

The equilibrium constants (K) and the Gibb's free energies ($\Delta G°$) are values at 293 K

phosphate. IMP could not be measured due to its limited aqueous solubility.

Effect of Phosphate Groups: Mono- and tri-phosphates. The introduction of two more phosphate groups at the 5'C position, i.e. forming GTP and ATP *vs.* GMP and AMP, enhanced complexation with norfloxacin. The slope of GTP's Stern-Volmer plot is steeper than that of GMP's (Figure 5(a)). Its equilibrium constant was *ca.* 4 × that of GMP, suggesting that norfloxacin interacted with phosphate groups, even in the absence of bridging Mg^{2+} ions. ATP also exhibited a larger equilibrium constant than AMP for association with norfloxacin, although the enhancement was less pronounced. The binding constant of ATP was 1.7 × that of AMP (Table 1). Figure 5(b) compares the van't Hoff plots for the complexation of norfloxacin with GMP and GTP. The slopes – i.e. enthalpy changes, $\Delta H°$ – are similar for GMP and GTP. The y-intercepts, denoting the entropy change, $\Delta S°$, are different; being negative for the formation of norfloxacin-GMP complexes, and slightly positive for the formation of norfloxacin-GTP complexes (Table 1). This indicates that the formation of GTP complexes was exothermic and entropically favorable. It also accounts for the largely enhanced equilibrium constant of the GTP complex. Similar result was observed for AMP and ATP: the increased

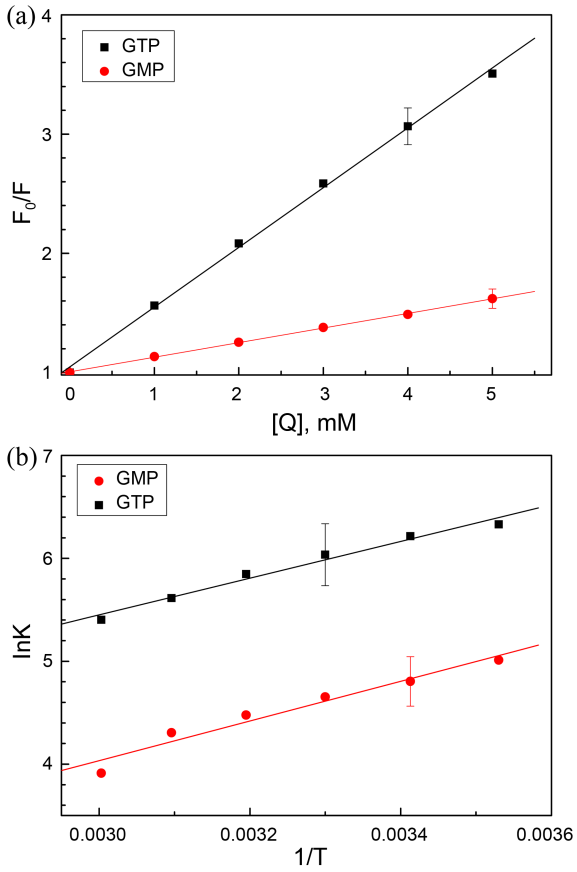

Figure 5. (a) Comparison of norfloxacin fluorescence quenching by GTP and GMP at 20 °C (b) Van't Hoff plots constructed from the temperature-dependent Stern-Volmer plots for norfloxacin-GTP and -GMP complex formation. Black squares denote GTP; red circles, GMP. Measurement conditions as per Figure 3.

presence of phosphate groups enhanced the binding affinity of the nucleotide to norfloxacin. AMP and ATP both showed negative enthalpy changes of complexation, with ΔH^o more negative for AMP. The entropy change of AMP complexation was negative while that of ATP complexation was positive, favoring the formation of norfloxacin-ATP complexes. The enhanced affinity for ATP was due to the favorable entropy change. The change of free energy of GTP complexation was slightly larger than that of ATP complexation. The van't Hoff plot for ITP is similar to that for ATP (Figure 6), suggesting similar thermodynamic parameters. ITP complexation was driven by both favorable entropy and enthalpy changes, similar to ATP complexation. Its free energy change and equilibrium constant were also similar. Therefore, that the equilibrium constant and free energy of GTP complexation were larger than those of ATP and ITP complexation may be related to the amine group at the C_2 position of the purine ring. Differences at the C_6 position, i.e. the presence of amine or carbonyl groups at this position, did not alter the binding affinity, or the free energy of complexation with norfloxacin.

Effect of Oxygen at the C'$_2$ Position: 2'deoxynucleotides. The fluorescence behaviors of norfloxacin with 2'deoxyAMP (dAMP) and 2'deoxyGMP (dGMP) are compared in Stern-Volmer (Figure 7(a)) and van't Hoff (Figure 7(b)) plots. dGMP showed a steeper positive slope in the van't Hoff plot than dAMP, suggesting that the complex formed between dGMP and norfloxacin was more stable than that with dAMP. The Stern-Volmer plots show two main differences. The quenching efficiencies of AMP and GMP were similar when oxygen was present at the C'$_2$ position, while the quenching of norfloxacin fluorescence by dGMP was *ca.* 4 ×

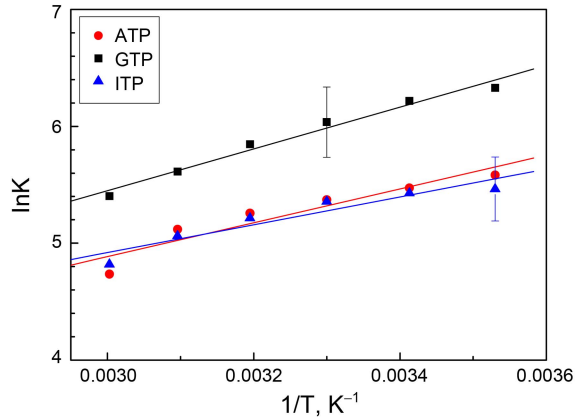

Figure 6. Van't Hoff plots for the association of norfloxacin with GTP (black squares), ATP (red circles) and ITP (blue triangles).

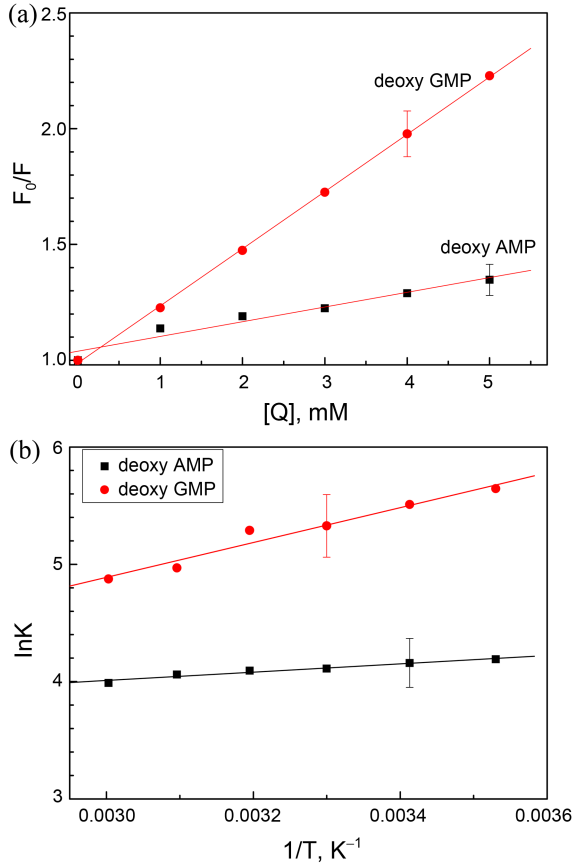

Figure 7. Effect of deoxygenation at the C'$_2$ position on (a) fluorescence quenching and (b) van't Hoff plots constructed from the temperature-dependent Stern-Volmer plots. Measurement conditions as per Figure 3.

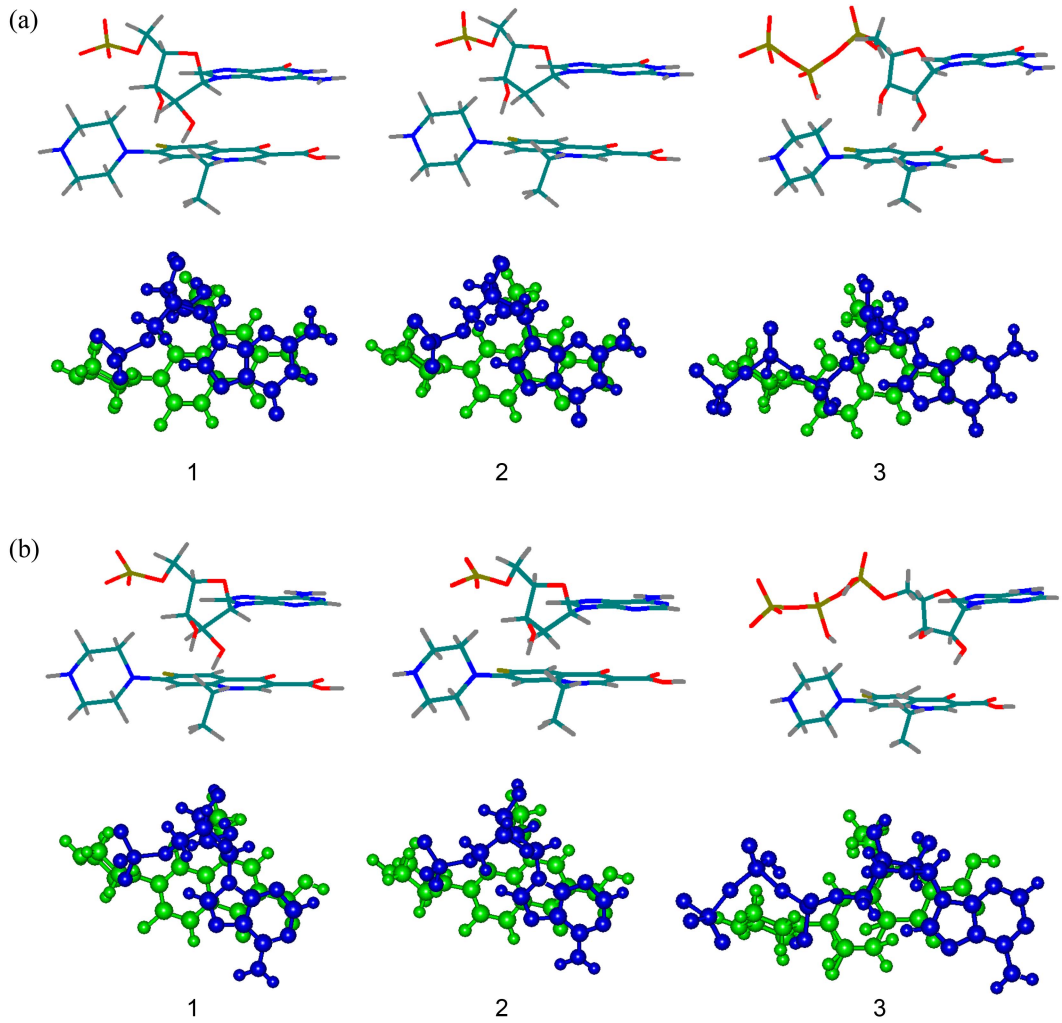

Figure 8. (a) Guanine nucleotides: (1) GMP, (2) dGMP and (3) GTP. (b) Adenine nucleotides: (1) AMP, (2) dAMP and (3) ATP. In the ball and stick models, norfloxacin and the nucleotides are colored blue and green, respectively.

more effective than by dAMP, suggesting more effective complexation with dGMP. Removal of the oxygen approximately doubled the equilibrium constant of GMP; it approximately halved that of AMP. The Gibb's free energy of complexation remained negative for both deoxiginated nucleotides; though the value for dGMP-norfloxacin complexation became more negative while that for dAMP decreased in magnitude. These differences in the behaviors of affinity and Gibb's free energy change were largely due to difference in the changes of enthalpy. Despite the entropy change being most favorable for dAMP, it showed a greatly reduced enthalpy change; hence it showed the smallest free energy change (Table 1). dGMP showed a slightly less favorable enthalpy change than GMP but this effect could be compensated by the large change in entropy. Removal of the oxygen atom resulted in the entropy change of complexation becoming positive but adversely affected the enthalpy change.

Discussion

These thermodynamic measurements show that differences between the DNA bases did not alter the binding affinity of norfloxacin to AMP or GMP. Complex formation was driven by favorable enthalpy changes; binding affinities increased when additional phosphate groups were introduced. The enhancement of equilibrium constant was more pronounced for GMP. IMP and AMP showed similar equilibrium constants, suggesting the importance of the amine group that protrudes from the minor groove of double-helical DNA. The additional phosphate groups resulted in the entropy changes of complexation becoming favorable, the main reason underlying the enhancement of equilibrium constant. Removing the oxygen atom from the C'_2 position resulted in enhanced complex formation for guanine, the binding affinity of adenine decreased. The stability of the dGMP complex was slightly reduced and the large favorable entropy change was responsible for the enhanced binding affinity. The stability of the adenine complex decreased greatly in spite of a very highly favorable entropy change. Complexes of norfloxacin and nucleotide formed spontaneously in all cases, with each showing negative free energy of formation.

Given these results, rational structures of the norfloxacin-nucleotide complexes could be constructed (Figure 8). The structures show the piperazine ring of the norfloxacin and the phosphate groups of the nucleotides in close proximity. They were likely stabilized by either electrostatic interactions or the formation of hydrogen bonds. The aromatic parts of norfloxacin and the DNA bases could stack with each other through π-π interactions. The complexes could be stabilized through both electrostatic attraction and hydrophobic interactions between the aromatic rings, which was conceivably responsible for the negative enthalpy change i.e., the exothermic formation. Increased presence of negatively charged phosphate groups greatly affected the entropy change of complexation, making it favorable, despite the decreasing number of species. The increased presence of negative charges may have resulted in stronger electrostatic interactions and so lowered the energy of the complexes. However the stabilization of the complexes that affected the enthalpy change, could not explain the entropy change. The change of ΔS^{o} was likely due to modification of the hydration shell, which may have become more disordered. The exact cause of the favorable entropy of complexation cannot be precisely explained from the current results and requires further study. Similar arguments may be applied to changes of ΔS^{o} for the complexing of dAMP and dGMP.

Conclusion

Thermodynamic properties for complex formation between norfloxacin and various purine nucleotides were investigated in this work. The formation of the complex was thermodynamically favorable with negative Gibb's free energy for all nucleotides. Increasing the number of phosphate group of nucleotides and removing the oxygen atoms at their C'_2 positions enhanced the formation of complexes between nucleotides and norfloxacin with the exception of dAMP. The enhancements for both cases were driven by favorable entropy changes. In the dAMP case, the favorable entropy change largely enhanced which was compensated by large decrease of the favorable enthalpy resulting in the smaller equilibrium constant.

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