Analysis of Binding Mode of 2'-GMP to Proteins Using $^1$H/$^{31}$P NMR Spectroscopy

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Abstract

$^1$H/$^{31}$P NMR techniques were applied to analyze the binding mode of guanosine 2’-monophosphate (2’-GMP) to histone. To date no structures of the complex comprising 2’-GMP and histone have been deposited in Protein Data Bank. Because $^{31}$P nucleus can be a selective marker of phosphorylated compounds, the combined use of $^1$H and $^{31}$P NMR spectroscopy has been applied to investigate these molecular interactions. The complex formation was initially confirmed by $^{31}$P-diffusion ordered spectroscopy and $^{31}$P-$T_1$ measurements. In the $^1$H-$^1$H saturation transfer difference experiments, H2’ and H3’ signals of 2’-GMP were significantly attenuated, while the rest of the unexchangeable protons were observed, indicating that contribution of H2’ and H3’ to the binding epitopes was low. The WaterLOGSY type experiment with $^{31}$P detection also indicated that a phosphorylated group located close to H2’ and H3’ had little access to histone.

Keywords Molecular interactions, Guanosine 2’-monophosphate, Histone, $^{31}$P NMR
Introduction

NMR spectroscopy is an important tool in the analysis of protein-ligand interactions for the elucidation of molecular recognition systems. Various NMR-based screening methods have been proposed to observe the $^1$H signals of ligands bound to proteins. These methods have included NOE-pumping, saturation transfer difference (STD), water-ligand observed via gradient spectroscopy (WaterLOGSY) and reverse NOE-pumping experiments. In the present study, the combined use of $^1$H and $^{31}$P NMR spectroscopy has been applied to investigate molecular interactions between guanosine 2’-monophosphate (2’-GMP) and histone. Because the $^{31}$P nucleus can be a selective marker of nucleic acids and other phosphorylated compounds, its observation is expected to provide useful information in this context, and $^{31}$P NMR spectroscopy can be a powerful tool for determination of phosphorus. Histone proteins provide critical structural support in the formation of chromosomes. Long DNA molecules need to fit into the cell nucleus, and they wrap around complexes of histone octamers in order to form more compact chromosomal shapes.

2’-GMP (Fig. 1) is a nucleotide containing one phosphorus atom and an ester of phosphoric acid with the nucleoside guanosine. Because no structures of the complex comprising 2’-GMP and histone have yet been deposited in the Protein Data Bank, the 2’-GMP-histone complex is of interest to study as a model system.

Experimental

Reagents and chemicals

2’-GMP and histone from calf thymus (type IIA) were purchased from Sigma-Aldrich. Three 600 μL solutions were prepared. The first solution contained 10 mM 2’-GMP and 0.4 mM histone...
prepared in 10% $^2\text{H}_2\text{O}$/90% $^1\text{H}_2\text{O}$. The other two solutions were prepared in 100% $^2\text{H}_2\text{O}$ and contained 10 mM 2'-GMP in the absence and presence of 62.5 μM–0.40 mM histone.

NMR spectroscopy

All NMR spectra were recorded at 20 °C on a Varian NMR-system 600 MHz equipped with a HFX probe. The experimental parameters for $^{31}\text{P}$-diffusion ordered spectroscopy (DOSY)$^8$ were as follows: data points, 4096; spectral width of $^{31}\text{P}$, 800 Hz; number of scans, 128; $\Delta = 0.1$ s; $\delta = 3.0$ ms; gradient field strength, 1.3-32 G/cm; increment, 15. The experimental parameters for $^1\text{H}$-DOSY were as follows: data points, 8192; spectral width of $^1\text{H}$, 8012 Hz; number of scans, 64; $\Delta = 0.05$ s; $\delta = 2.0$ ms; gradient field strength, 1.3-32 G/cm; increment, 15. The experimental parameters for $^{31}\text{P}$ spin-lattice relaxation times ($T_1$) were as follows: data points, 2048; spectral width of $^{31}\text{P}$, 800 Hz; number of scans, 16; relaxation delay, 16 s. The inversion recovery pulse sequence was used. Variable delays between 180° and 90° pulses were 0.0625, 0.125, 0.25, 0.5, 1.0, 2.0, 4.0 and 8.0 s. In the $^{31}\text{P}$-WaterLOGSY and $^{31}\text{P}[^1\text{H}]$ STD experiments, the same pulse sequence was used. The common experimental parameters of these experiments were as follows: data points, 4096; spectral width of $^{31}\text{P}$, 800 Hz; number of scans, 10240; $^1\text{H}$ irradiation time, 3.0 s. The $^1\text{H}$ selective irradiation was set to the water resonance in the $^{31}\text{P}$-WaterLOGSY experiment. The $^1\text{H}$ selective irradiation was set to the water resonance in the $^{31}\text{P}$-WaterLOGSY experiment. The experimental parameters for $^1\text{H}[^1\text{H}]$ STD experiment were as follows: data points, 16384; spectral width of $^1\text{H}$, 8012 Hz; number of scans, 64. Those of $^1\text{H}[^1\text{H}]$ STD experiment for the STD effect were as follows: data points, 16384; spectral width of $^1\text{H}$, 8012 Hz; number of scans, 512; saturation times, 0.5-3.5 s. The on- and off-resonance frequencies of $^1\text{H}$ were 0.6 and -20 ppm, respectively, in both $^{31}\text{P}[^1\text{H}]$ and $^1\text{H}[^1\text{H}]$ STD experiments. The $^1\text{H}[^1\text{H}]$ STD for the STD effect,
Results and Discussion

The binding of 2′-GMP to histone was initially confirmed by 31P-DOSY experiments, which utilized the observed diffusion coefficients to measure the extent of bound and unbound states in equilibrium. As the diffusion coefficient of a molecule is inversely proportional to its molecular radius, a decrease in this measure reflects an increase in bound states. The diffusion coefficients of 31P, corresponding to those of 2′-GMP, gradually decreased as the molecular excess of 2′-GMP was reduced (Table 1). This decreased diffusion was attributed to the complex formation leading to increases in both the size and molecular weight. The dissociation constant ($K_d$) of 2′-GMP was calculated based on the diffusion coefficient of the sample containing 0.125 mM histone and 10.0 mM 2′-GMP (1:80), where the diffusion coefficient of the free histone was measured at 0.59 ± 0.006 ($\times 10^{-10}$ m$^2$/s) by 1H-DOSY experiments. Assuming a model for 1:1 binding equilibria in fast exchange, $K_d$ was calculated to be 7.7 mM. The 31P-$T_1$ values reflected a tendency similar to the diffusion coefficients of 31P (Table 1). The 31P-$T_1$ values also decreased as the molecular excess of 2′-GMP was reduced. Specifically, a decrease of $T_1$ indicates an increase in molecular correlation time in the small molecules such as 2′-GMP, and thus a decrease in molecular diffusion. In particular, because variations of signal intensities were observed in both DOSY and $T_1$ measurements, observations of 31P signals, which do not overlap with protein resonances, were advantageous in the accurate analyses of the macromolecular complexes.

The 1H{1H} STD spectrum of 2′-GMP in the presence of histone is shown in Fig. 2a. The 1H
resonance at $\delta^1\text{H} = 0.6$ ppm, corresponding to a methyl region, was selectively irradiated as on-resonance frequency, and the irradiation at $\delta^1\text{H} = -20$ ppm was performed for reference. Several signals in the $^1\text{H}\{^1\text{H}\}$ STD spectrum indicated a binding of 2'-GMP to histone (Fig. 2a). In particular, the signals of H8, H1’, H4’ and H5’ in 2'-GMP were clearly observed, whereas those of H2’ and H3’ were not, indicating that little saturation transfer occurred between the methyl protons of histone to H2’ and H3’ of 2'-GMP. These two protons in the pentose sugar of the nucleotide are located near the phosphate group (Fig. 1), implying that the phosphate group blocks the access of H2’ and H3’ to histone. To confirm the binding epitopes of 2’-GMP, $^1\text{H}\{^1\text{H}\}$ STD experiments at various saturation times were carried out, and the STD build-up curve was obtained for each $^1\text{H}$ signal of 2’-GMP as shown in Fig. S1 (Supporting Information). The slope at a saturation time of 0 s, called the STD effect, reflects the proximity of ligand protons to receptor protons, and was calculated by fitting. The values of the STD effect are shown in Table 2. These values were normalized by referencing to the signal of H5’ with the largest STD effect as 100%. The STD effects of H8, H1’ and H4’ were relatively similar, whereas those of H2’ and H3’ could not be determined owing to their attenuated intensities.

The $^{31}\text{P}$-WaterLOGSY experiments were performed to confirm the binding contribution of the phosphate group. The pulse sequence of $^{31}\text{P}$-WaterLOGSY was conceptually identical to that of $^{19}\text{F}$-WaterLOGSY, where $^{19}\text{F}$ channel was switched to $^{31}\text{P}$. In the $^{31}\text{P}$-WaterLOGSY, the $^1\text{H}$ magnetization of water, which was transferred to the protein, was again transferred to $^{31}\text{P}$ of the bound ligand via direct or indirect relay processes. Although the binding of 2’-GMP to histone was confirmed by $^1\text{H}\{^1\text{H}\}$ STD, $^{31}\text{P}$-DOSY and $^{31}\text{P}$–$T_1$ experiments, the $^{31}\text{P}$ signals of 2’-GMP in
$^{31}$P-WaterLOGSY spectra were unexpectedly observed in the same phase in the presence and absence of histone (Fig. 3c,d), despite being expected to be in opposite phases. It can be considered that ineffective saturation transfer to H2' and H3' interrupted further magnetization transfer to $^{31}$P of 2'-GMP. In addition, the direct transfer of $^1$H magnetization of water to $^{31}$P of 2'-GMP in the unbound state could be dominant, resulting in this observed anomaly. Although $^{31}$P/$^1$H STD experiments were carried out, in which $^1$H resonance of methyl region of histone was selectively irradiated, no $^{31}$P signals were observed (data not shown). This result indicated that the binding contribution of the phosphate group to the methyl group of histone was minimal.

**Conclusion**

It was demonstrated that the combined use of $^1$H/$^{31}$P NMR spectroscopy provided valuable information in analyses of molecular interactions at the atomic and molecular levels. Because the $^{31}$P signals were not overlapped with the protein and water signals, its observation was useful for the accurate measurements of the signal intensity changes in DOSY and $T_1$ experiments. $^{31}$P nucleus can be a selective marker of phosphorus-containing compounds, which were advantageous in observing protein-nucleic acid complex using NMR spectroscopy.

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**Supporting Information:** The STD build-up curve for each $^1$H signal of 2'-GMP. This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.
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Table 1  $^{31}$P diffusion coefficients and $^{31}$P-$T_1$ of 2'-GMP at various molar ratio.

| Sample (histone : 2'-GMP)\(^a\) | $^{31}$P diffusion coefficients (x$10^{-10}$ m$^2$/s) | $^{31}$P-$T_1$ (s) |
|---------------------------------|---------------------------------|-----------------|
| 10 mM 2'-GMP                   | 3.75±0.12                       | 1.77±0.05       |
| 1 : 160                         | 3.43±0.15                       | 1.69±0.03       |
| 1 : 80                          | 3.22±0.08                       | 1.61±0.02       |
| 1 : 50                          | 2.99±0.24                       | 1.49±0.08       |
| 1 : 25                          | 2.66±0.07                       | 1.23±0.04       |

\(^a\) A concentration of 2'-GMP was fixed to 10 mM in each solution.
Table 2  $^1$H-STD effects (STD$_{eff}$) of 10 mM 2'-GMP in the presence of 0.4 mM histone.

|     | H8   | H1'  | H2$^a$ | H3$^a$ | H4'  | H5'  |
|-----|------|------|--------|--------|------|------|
| STD$_{eff}$ | 70.8±0.5 | 62.9±1.7 | –      | –      | 78.5±2.4 | 100  |

$^a$ $^1$H-STD effects of H2’ and H3’ could not be determined owing to the attenuated intensities.
Figure captions

Fig. 1 Structure of 2’-GMP.

Fig. 2 (a) $^1$H{$^1$H} STD and (b) $^1$H spectra acquired using a solution containing 0.4 mM histone and 10 mM 2’-GMP. In (a), STD pulse sequence incorporating a WET sequence was used to observe the ligand signals resonated close to water.  

Fig. 3 $^{31}$P spectra acquired using solutions containing (a) 0.4 mM histone and 10 mM 2’-GMP, and (b) 10 mM 2’-GMP. $^{31}$P-WaterLOGSY spectra acquired using solutions containing (c) 0.4 mM histone and 10 mM 2’-GMP, and (d) 10 mM 2’-GMP.
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Graphical Index