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Quantitative analysis of the location- and sequence-dependent deamination by APOBEC3G using real-time NMR**

Ayako Furukawa, Kenji Sugase, Ryo Morishita, Takashi Nagata, Tsutomu Kodaki, Akiti Takaori-Kondo, Akihide Ryo, and Masato Katahira*
location-dependent deamination. Although catalytically inactive CD1 also supposedly contributes to the location-dependent deamination, we assumed that the intrinsic characteristics of CD2 are important for the location-dependent deamination by full-length A3G. We focused on CD2 in the following experiments. Subsequently, we investigated whether or not the real-time NMR method can be used to examine A3G sliding along ssDNA. Since A3G binds to ssDNA but not to dsDNA, [10] intervening dsDNA should block A3G sliding. As shown in Figure 1c and d, the deamination rates of two hot spots in a substrate with short dsDNA between them were almost the same, whereas the rates were location-dependent without dsDNA (SCCCCCCC and SCCCCCCC in Table 1). This finding clearly indicates that real-time NMR can sense A3G CD2 sliding along ssDNA.

Table 1. Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Name</th>
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<tr>
<td>SCCCC</td>
<td>ATTCGCCCATTTTTTTTTATCCCGATTT</td>
</tr>
<tr>
<td>SCCCCCCC</td>
<td>ATTCGCCCATTTTTTTTTTTTTTTTTTATCCCGATTT,3</td>
</tr>
<tr>
<td>SCCCCCCC</td>
<td>ATTCGCCCATTTTTTTTTTTTTTTTTTTTTTTATCCCGATTT,3</td>
</tr>
<tr>
<td>SCCCC</td>
<td>GCCTTGGCGGTTCCCC</td>
</tr>
<tr>
<td>SCCCC</td>
<td>TTAACCATTAT</td>
</tr>
<tr>
<td>SCCCC</td>
<td>TTAACCATTAT</td>
</tr>
<tr>
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<td>ATTCGCCCATTT</td>
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The cytidines that are deaminated are underlined.

We confirmed that real-time NMR is sufficiently sensitive to the location-dependent deamination and sliding processes of A3G CD2. Based on the results, we designed experiments and constructed a kinetic model to quantitatively analyze the deamination reaction. Since A3G binds to ssDNA nonspecifically, the binding rate should depend on the length of the ssDNA, and the concentrations of A3G and ssDNA. On the other hand, the sliding duration to reach a hot spot should depend on the length of the ssDNA and the position of a hot spot. Therefore, the kinetics of the deamination can be characterized by performing real-time NMR experiments with different concentrations of ssDNA and A3G CD2, different lengths of ssDNAs, and different positions of hotspots. According to this idea, we developed a theoretical kinetic model for the analysis of real-time NMR data (Supporting Information). The deamination reaction monitored by NMR as the change of the intensity, \( I(t) \), is expressed as:

\[
I(t) = I_0 \exp(-k_{\text{total}} t)
\]

where \( I_0 \) is the initial intensity, proportional to the concentration of ssDNA, and \( k_{\text{total}} \) represents the apparent deamination rate calculated as below:

\[
k_{\text{total}} = \frac{1}{S_k \mathcal{K}_d \mathcal{K}_c} \mathcal{E} \left[ \mathcal{K}_d(3'\rightarrow5') + \mathcal{K}_c(5'\rightarrow3') \right] \]

\[
\mathcal{S} = \frac{1}{2} \mathcal{N} \mathcal{E} \left[ \mathcal{K}_d(3'\rightarrow5') + \mathcal{K}_c(5'\rightarrow3') \right]
\]

\[
\mathcal{E} = \frac{1}{2} \mathcal{N} \mathcal{E} \left[ \mathcal{K}_d(3'\rightarrow5') + \mathcal{K}_c(5'\rightarrow3') \right]
\]

\[
a = k_1 \left( k_2 + k_3 \right)
\]

\[
br = 2 \left( \mathcal{N} + 1 \right) \left( 1 - \mathcal{N} \alpha \right)
\]

\[
\alpha = k_1 \left( k_2 + k_3 \right)
\]

\[
\beta = k_4
\]

The cytidines that are deaminated are underlined.

Figure 1. Real-time monitoring of deamination reactions at two CCC hot spots in an ssDNA. (a, b) The deamination reactions of full-length A3G (a) and CD2 (b) were monitored for C6 (red) and C22 (blue) in SCCCC. The deamination rates for full-length A3G are 1.3×10^{-6} ± 4.0×10^{-8} s^{-1} for C6 and 4.1×10^{-6} ± 7.4×10^{-8} s^{-1} for C22, and those for CD2 are 2.0×10^{-4} ± 7.5×10^{-6} s^{-1} for C6 and 4.2×10^{-5} ± 5.0×10^{-7} s^{-1} for C22. (c, d) The deamination reactions of CD2 were monitored for C6 (purple) and C33 (yellow) in SCCCCCCC (c) and SCCCCCCC (d).
has of being detected by A3G approaching from downstream than from upstream; therefore a CCC hot spot located close to the 5’ end is deaminated more rapidly than one less close to the 5’ end.

Figure 2. Quantitative analysis of the location-dependent-deamination reaction using the real-time NMR. (a) A kinetic model of A3G deamination. (b) The real-time NMR data for C6 of SCCC (red), C26 of SCCC (green), C45 of SCCC (blue), and both C6 (purple) and C33 (yellow) of SCCCCC were fitted to equation (1).

For further applications of our real-time NMR, we analyzed the deamination of CCCC, in which two CCC sequences tandemly overlap (SCCCC in Table 1). The clusters of 3-6 consecutive cytidines are scattered on the HIV genome, and CCCC is the second most abundant after CCC. Unfortunately, NMR peaks overlapped during the deamination reactions, for example, the TOCSY peaks of the fourth cytidines of CCCC and CCUC. Thus, the time course of each deamination reaction could not be monitored separately (Figure 3a and b). However, we characterized the deamination reactions using the NMR spectrum of the same ssDNA whose deamination reactions were fully completed. As a result, the third and fourth cytidines of CCCC were found to be deaminated with equal efficiency by A3G CD2 because the intensities of peaks 2 (CCUC) and 3 (CCCU+CCUU) were nearly identical. This finding is different from that of a previous study by gel shift assay; the third cytidine was deaminated more efficiently than the fourth. This discrepancy could be caused by the difference in the monitoring durations between our NMR method (6 hours) and the gel shift assay (3 minutes). The real-time NMR can monitor multiple reactions directly and simultaneously using a single sample, whereas the gel shift assay cannot. Therefore, our results should provide more accurate information.

The deamination of CCCU into CCUU is redundant and may be unnecessary for abolition of the HIV infectivity. However, the above experiment could not provide information on the deamination efficiency as to CCCU because the NMR peaks of CCCU and CCUC overlapped. Thus, we monitored the deamination reactions as to CC CA (undeaminated site) and CC CU (singly deaminated site) to determine whether or not A3G has a preference for either of the two sequences, SCCCA and SCCCU (Table 1). In this case, the deamination reactions were monitored by one-dimensional 1H NMR to increase the spectral resolution (Figure 3c and d). The results revealed that A3G preferentially deaminates an undeaminated site rather than a singly deaminated site because the intensity of the deaminated product CCUA (peak 4) was higher than that of CCUU (peak 3). A3G would exploit this sequential preference to efficiently deaminate multiple hotspots scattered on the HIV genome with little redundant deamination of CCCC.

In conclusion, we analyzed the real-time NMR data for the location-dependent deamination by A3G CD2 using the newly derived kinetic model. As a result, the location-dependent deamination can be explained by the difference in $k_{cat}$ of CD2 depending on the direction of approach to the target cytidine. We also characterized the deamination reactions as to CCCC using the real-time NMR even though multiple reactions occurred simultaneously. Previously, it was proposed that the location-dependent deamination is caused by two binding orientations on ssDNA and the existence of a deamination dead-region (~30 nucleotides) at the 3’ end of the ssDNA. However, this model cannot explain the location-dependent deamination observed for a long ssDNA (70 nucleotides) because the contribution of the dead-region to the location-dependency becomes smaller. On the other hand, our model can explain the location-dependent deamination even for such a long ssDNA.

Recently, it was proposed that APOBEC family proteins are involved in epigenesis. They may contribute to the removal of an epigenetic marker, a methyl group of a 5-methyl cytosine, through deamination of a 5-hydroxymethyl cytosine. Our real-time NMR method can also be applied to examine such DNA modifications and provide insights into epigenesis. Furthermore, this method can also be used for the analysis of post-translation modifications of proteins.
Keywords: Real-time NMR • location-dependent deamination • quantitative analysis • Enzyme kinetics • APOBEC3G

Quantitative analysis of the location- and sequence-dependent deamination by APOBEC3G using real-time NMR

APOBEC3G (A3G) efficiently deaminates cytidines located close to the 5’ end of single-stranded minus DNA of the HIV-1 genome. However, this mechanism is elusive because no suitable method exists to analyze the deamination process that includes A3G non-specific binding to DNA and sliding along it. Here, we quantitated this process using a newly developed real-time NMR method. As a result, the location-dependent deamination can be explained by two catalytic rates depending on the direction of approach to the target cytidine.
Supporting Information

Materials and methods

Preparation of A3G

The cDNAs of full-length and CD2 A3G were inserted into the pEU-E01-GST-ps-Flag expression vector (Cell Free Sciences). Full-length A3G with a GST tag was synthesized using the wheat germ cell-free protein expression system as described previously. The synthesized protein was loaded onto a Glutathione-Sepharose resin column (GE Healthcare), and the GST tag was cleaved with TEV protease on the column. A3G was eluted from the column with PBS (pH 7.4) containing 5 mM DTT, 10 µM ZnCl₂, and 0.04 % Briji35. The protein solution was concentrated using an ultrafiltration cartridge (Millipore). For A3G CD2, the protein was expressed and purified as described previously. After purification, the protein was dialyzed against the same buffer as that for full-length A3G and concentrated.

Monitoring of deamination using NMR

Each DNA purchased from Fasmac was dissolved in PBS (pH 7.4) containing 5 mM DTT, 10 µM ZnCl₂, and 0.04% Briji35 at a concentration of 220 µM. The molar ratios of A3G:ssDNA were 1:44 (Figure 1a,b), 1:100 (Figure 1c,d and 2), and 1:5 (Figure 3). After the addition of A3G, a series of ¹H one- and two-dimensional TOCSY spectra of DNA were recorded with a mixing time of 20 ms at 25 ºC to monitor the deamination in real-time on a Bruker DRX 600 NMR spectrometer equipped with a cryoprobe and a Z-gradient (Bruker Biospin). The experimental error was estimated from the noise level of the TOCSY spectra, and the real-time NMR data were fitted to \( I(t) = I₀\exp(−k_{deami}t) \) to derive an apparent
deamination rate, $k_{\text{deami}}$.

**Derivation of a theoretical kinetic model for the analysis of real-time NMR data**

A general enzyme reaction is expressed as:

$$E + S \leftrightarrow ES \rightarrow EP \rightarrow E + P$$

where $E$, $S$, and $P$ represent enzyme, substrate and product, respectively. The enzyme-substrate complex ES completely changes into the enzyme-product complex EP, provided that the enzyme dissociates from the substrate much slower than the catalytic reaction occurs. The enzyme does not rebind to the product. In the case of A3G, however, the deamination reaction does not always occur even if A3G binds to ssDNA because A3G can form non-active complexes with ssDNA.\(^3\)\(^4\) In our kinetic model, A3G binds to any nucleotide of ssDNA at binding rate $k_{\text{on}}$, and dissociates from ssDNA at dissociation rate $k_{\text{off}}$, because A3G exhibits no sequential preference for binding.\(^5\) This model also includes multiple separate interactions between A3G and ssDNA. As explained in the main text, our model assumes that A3G slides along ssDNA in both directions at sliding rate $k_s$, without a change in direction during a single sliding event. The catalytic rate is defined as $k_{\text{cat}(3'\rightarrow5')}^{\text{catalytic}}$ when A3G approaches a target cytidine from downstream, whereas it is $k_{\text{cat}(5'\rightarrow3')}^{\text{catalytic}}$ when A3G approaches from upstream (Figure 3a). Although A3G cannot deaminate a single cytidine, we first describe a hypothetical case where A3G can deaminate a single cytidine, and the total number of nucleotides in ssDNA is set to 3 for simplicity. Namely, A3G deaminates cytidines in the sequences Cxx xCx, and xxC (x is a nucleotide other than cytidine). The theoretical time courses of the intensities of cytidines located at the first, second, and third positions from the 3' end, $I_i(t)$ ($i=1, 2, 3$) are expressed as follows, respectively:
where $I_0$ is the initial intensity, proportional to the total concentration of ssDNA. $[ES_1]$, $[ES_2]$, and $[ES_3]$ are the concentrations of ssDNA bound to A3G under the condition that A3G slides toward the 5’ end. $[ES_1']$, $[ES_2']$, and $[ES_3']$ are the same as $[ES_1]$, $[ES_2]$, and $[ES_3]$ except that A3G slides toward the 3’ end. Subscripts indicate the positions of the cytidines from the 3’ end. $[ES_i]/S_0 (i=1, 2, 3, 1’, 2’, 3’)$ represents the fractional population of each A3G:ssDNA complex. When the concentrations of free ssDNA, free A3G and total A3G are defined as $[S]$, $[E]$, and $E_0$, respectively,

$$[S]+[ES_1]+[ES_2]+[ES_3]+[ES_1]+[ES_2]+[ES_3]=S_0$$  \hspace{2cm} (S2)$$

$$[E]+[ES_1]+[ES_2]+[ES_3]+[ES_1]+[ES_2]+[ES_3]=E_0$$  \hspace{2cm} (S3)$$

We assume that A3G interacts with the products (Uxx, xUx, and xxU) at the same $k_{on}$ and $k_{off}$ as for the substrates. Then, the concentration of A3G at each position of ssDNA is held in equilibrium even if the cytidine is deaminated. In this case, the concentrations in (6) are derived by solving the following differential equation under the condition that $dC/dt=0$: 

$$I_1(t) = I_0 \exp \left\{ - \left[ \frac{[ES_1]}{S_0} k_{cat(3’\rightarrow 5’)} + \frac{[ES_1]}{S_0} k_{cat(5’\rightarrow 3’)} \right] t \right\}$$

$$I_2(t) = I_0 \exp \left\{ - \left[ \frac{[ES_2]}{S_0} k_{cat(3’\rightarrow 5’)} + \frac{[ES_2]}{S_0} k_{cat(5’\rightarrow 3’)} \right] t \right\}$$

$$I_3(t) = I_0 \exp \left\{ - \left[ \frac{[ES_3]}{S_0} k_{cat(3’\rightarrow 5’)} + \frac{[ES_3]}{S_0} k_{cat(5’\rightarrow 3’)} \right] t \right\}$$

(S1)
\[
\frac{d}{dt} C = KC
\]

\[
C = \begin{bmatrix}
\end{bmatrix}^T
\]

\[
K = \begin{bmatrix}
-6[E]k_{on} & k_{off} & k_{off} & k_{off} + k_s & k_{off} + k_s & k_{off} & k_{off} \\
k_{on} & -k_{off} - k_s & 0 & 0 & 0 & 0 & 0 \\
k_{on} & k_s & -k_{off} - k_s & 0 & 0 & 0 & 0 \\
k_{on} & 0 & k_s & -k_{off} - k_s & 0 & 0 & 0 \\
k_{on} & 0 & 0 & k_s & -k_{off} - k_s & 0 & 0 \\
k_{on} & 0 & 0 & 0 & k_s & -k_{off} - k_s & 0 \\
k_{on} & 0 & 0 & 0 & 0 & k_s & -k_{off} - k_s \\
\end{bmatrix}
\]

(S4)

Simple mathematical manipulation together with (7) and (8) provides,

\[
[ES_1] = [ES_2] = [E][S](1 - \alpha)/K_d
\]

\[
[ES_2] = [ES_1] = [E][S](1 + \alpha)(1 - \alpha)/K_d
\]

\[
[ES_2] = [ES_1] = [E][S](1 + \alpha^2)(1 - \alpha)/K_d
\]

(S5)

\[
[S] = \frac{1}{2}(-\beta + S_0 - E_0 + \sqrt{(\beta - S_0 + E_0)^2 + 4E_0\beta})
\]

(S6)

\[
[E] = \frac{1}{2}(-\beta - S_0 + E_0 + \sqrt{(\beta + S_0 - E_0)^2 + 4E_0\beta})
\]

(S7)

\[
\alpha = k_s/(k_{off} + k_s), \quad \beta = \frac{K_d}{2\{3 + 1/(1 - \alpha^3)}(1 - \alpha)\}
\]

(S8)

, where \(K_d\) is the dissociation constant \((=k_{off}/k_{on})\). By substituting (10) for (6),

\[
I_1(t) = I_0 \exp\left\{-\frac{[E][S](1 - \alpha)}{S_0 K_d} \left[k_{\text{cat}(3\rightarrow5)} + k_{\text{cat}(5\rightarrow3)} \left(1 + \alpha + \alpha^2\right)\right]t\right\}
\]

\[
I_2(t) = I_0 \exp\left\{-\frac{[E][S](1 - \alpha)}{S_0 K_d} \left[k_{\text{cat}(3\rightarrow5)}(1 + \alpha) + k_{\text{cat}(5\rightarrow3)}(1 + \alpha)\right]t\right\}
\]

\[
I_3(t) = I_0 \exp\left\{-\frac{[E][S](1 - \alpha)}{S_0 K_d} \left[k_{\text{cat}(3\rightarrow5)}(1 + \alpha + \alpha^2) + k_{\text{cat}(5\rightarrow3)}\right]t\right\}
\]

(S9)

In the case where the total number of nucleotides is \(N\),

\[
N_{I_1}(t) = I_0 \exp\left\{-\frac{[E][S](1 - \alpha)}{S_0 K_d} \left[k_{\text{cat}(3\rightarrow5)}(1 + \alpha + \alpha^2 + \cdots + \alpha^{N-4}) + k_{\text{cat}(5\rightarrow3)}(1 + \alpha + \alpha^2 + \cdots + \alpha^{N-4})\right]t\right\}
\]

\[
N_{I_2}(t) = I_0 \exp\left\{-\frac{[E][S](1 - \alpha)}{S_0 K_d} \left[k_{\text{cat}(3\rightarrow5)}(1 - \alpha^4) + k_{\text{cat}(5\rightarrow3)}(1 - \alpha^{N-1})\right]t\right\}
\]

(S10)

S4
\[
\begin{align*}
[\text{N}] & = \frac{1}{2} \left( -\beta^N + S_0 - E_0 + \sqrt{\left(\beta^N - S_0 + E_0\right)^2 + 4E_0 \beta^N} \right) \\
[\text{E}] & = \frac{1}{2} \left( -\beta^N - S_0 + E_0 + \sqrt{\left(\beta^N + S_0 - E_0\right)^2 + 4E_0 \beta^N} \right)
\end{align*}
\]

\[
\beta^N = \frac{K_d}{2\left(N + 1 - (1 - \alpha)^N\right)/(1 - \alpha)}
\]

The superscripts \(N\) and \(n\) indicate the total number of nucleotides of ssDNA and the position of the reacting cytidine from the 3’ end of ssDNA, respectively.

**Chemical shift assignments of the cytidines and uridines in Figure 3a and b**

TOCSY spectra of \(S_{\text{CCCC}}\) were recorded at 0 and 6 hours after the addition of A3G CD2. To assign peaks 1-4, TOCSY spectra of \(S_{\text{CCCU}}\) and \(S_{\text{CCUC}}\) were also recorded (Figure S2). Through comparison between Figure S2a and c, peak 1 was assigned to the fourth cytidine of \(\text{CCCC}\) (corresponding cytidine or uridine is underlined). The intensity of peak 2 did not change any more after 6-hour incubation, and thus the reactions \(\text{CCCC} \rightarrow \text{CCCU}\) and \(\text{CCCC} \rightarrow \text{CCUC}\) should have been completed. Therefore, peak 2 was assigned to the fourth cytidine of the \(\text{CCUC}\). Through comparison between Figures S2b and c, and between Figure S2b and d, peaks 3 and 4 were assigned to the fourth uridine of \(\text{CCCU}\) and the third uridine of \(\text{CCUC}\), respectively. Peak 4 is broad, and thus it should include the correlation peak for the third uridine of \(\text{CCUU}\). Similarly, peak 3 should contain the correlation peak of \(\text{CCUU}\) because of its structural similarity to \(\text{CCUC}\). The absence of broadening for peak 3 implies that the chemical shifts for these two peaks are virtually identical.
Figure S1. Model for the sliding-direction-dependent binding to the catalytic pocket.

(a) Overall structure of A3G CD2 (PDB:3E1U) and a closed view of the catalytic pocket as a surface representation. The catalytic pocket includes Glu259 (blue) and a zinc ion (red), which are important for the deamination reaction. The putative bump is highlighted by a bold line. (b) Schematic diagrams of sliding-direction-dependnet entry of cytidine into the catalytic pocket. Sliced views at the yellow arrow in
(a) are illustrated. The hollow cavity and the bump in A3G are depicted in gray and orange, respectively. The ssDNA backbone and cytidines are depicted as dark green lines and light green hexagons, respectively. Black arrows indicate the direction of A3G sliding. When A3G approaches a CCC hot spot from downstream (3’), the third cytidine of CCC can be ideally accommodated into the catalytic pocket for the deamination reaction (upper panel). On the other hand, when A3G approaches from upstream (5’), the third cytidine encounters a bump before reaching the pocket. Thus, the cytidine needs to overcome the bump to reach the pocket, causing overrunning of the cytidine. In this case, the cytidine cannot be ideally accommodated in the catalytic pocket (bottom panel), resulting in reduction of the catalytic rate.
Figure S2. The chemical shifts assignments of the deamination products of CCCC.

The H5-H6 TOCSY spectrum of $S_{CCC}$ (a) and that at six hours after the addition of A3G CD2 (b). The molar ratio of A3G:$S_{CCC}$ was 1:5. The H5-H6 TOCSY correlation peaks of $S_{CCCU}$ (c) and those of $S_{CCUC}$ (d). The peaks are labeled with the sequence of the corresponding substrate or deaminated product in which the assigned residue is shown in underline.
REFERENCES


