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Effects of Asymmetric Arginine Dimethylation on RNA-Binding Peptides

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Arginine is the most frequent methylation site (about 90%) in proteins found in higher eukaryotes.[1] Numerous studies have shown that Arg methylation in RNA-binding proteins (RBP) can regulate pre-mRNA processing[2] and control the stability of mRNA through modulation of both protein–protein and protein–RNA interactions.[3] Many RBP's,[4] as well as proteins involved in transcription and RNA metabolism,[5] serve as substrates for the enzyme Arg methyl transferase (PRMT). Since Arg methylation can be reversed by enzyme catalyzed demethylation,[6] the reversible modification plays a critical role as a gene-control mechanism.[7]

The role(s) of Arg methylation of proteins, however, remains an intriguing question, especially with regard to molecular recognition of RNA, even though it has both positive and negative effects on protein–protein interactions.[2] The results of previous studies with a small number of synthetic Arg methylated peptides led to the conclusion that methylation does not promote significant changes in binding affinities to RNA.[8] Also, in biological experiments, no significant physiological changes[9] or reductions[2,10] in binding affinities with RNA were observed upon Arg methylation. However, these investigations employed indirect methods, utilized random sequences from the repeated sequence Arg-Gly-Gly found in nuclear and hnRNP proteins (RGG box), and involved comparisons of properties of the native proteins with those of Lys and Ala mutants.[2,10] Consequently, the findings and conclusions might have misrepresented the role that methylation plays in interactions between proteins and RNA.

To analyze the physicochemical changes that arise from alterations in the affinities of methylated proteins (or peptides) for their cis-element RNA, we thought that a large number of modified proteins (or peptides) should be probed. The Rev peptide was chosen as a model for this purpose, because it contains ten Arg residues in its RNA-binding motif. The well known RBP, Rev, is a key peptide in HIV propagation. When unspliced or partially spliced viral RNA is imported into the nucleus, Rev binds to the Rev responsive element (RRE) in the RNA of HIV-1 and increases gene transcription.[10] Furthermore, the Rev peptide has α-helical structures, which provide opportunities to explore the effects of methylation on conformational rigidity.

The effects of methylation might depend on the position[11] and number of asymmetric dimethylated Arg residues, which result from the operation of a major methylation mode.[1,12] A recent report suggested that Rev is a substrate for asymmetric dimethylation.[10] But physicochemical effects of methylation on the individual Arg residues have not been demonstrated yet.

In this study, all possible Rev mutant peptides containing asymmetric dimethylated Arg residues[13] were synthesized and their binding affinities to RRE were measured. The results, described below, demonstrate that a variety of binding affinity changes take place upon methylation of the Arg residues of Rev; these range from a 25-fold reduction to a 1.3-fold increase in binding affinity to RRE RNA. The findings suggest that the affinity changes brought about by methylation are highly dependent on the position of the Arg residue, and lead to the interesting conclusion that post-translational methylation could be used process as a reversible switch to differentiate protein–RNA interactions in a selective manner.

A library of peptides was synthesized by using the standard Fmoc solid-phase protocol. The dissociation constant (K_d) for the binding of each peptide to RRE was measured[14] by using the electrophoretic gel mobility shift assay (Figure 1, Table 1). As expected, modifications of Arg residues that have been identified to have the most contact with RRE based on the so-

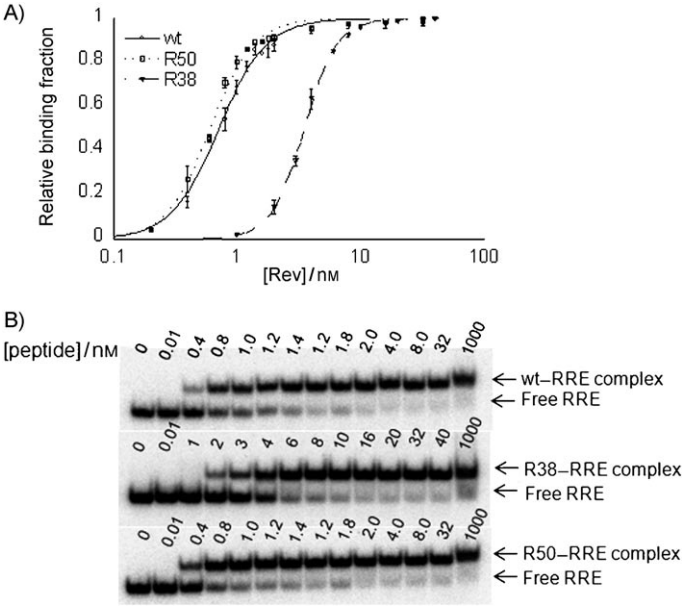

Figure 1. Selected EMSA analysis of Rev peptides that bound to RRE. A) Plot of the binding fraction against the concentration of Rev peptide (0–1000 nM); RRE (1 nM) was mixed with increasing amounts of Rev peptide. B) A representative EMSA assay in which RRE (1 nM) was mixed with increasing amounts of Rev; wt: wild-type protein.

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Table 1. K_d values[a] of Rev peptides against RRE RNA and their helicities in the absence and presence of RNA.[14]

Rev peptide[b]	K_d [nM][c]	α Helicity [%]	α Helicity with RRE [%]
wild-type	0.77 ± 0.12	33	85
R35	0.63 ± 0.062	36	100
R38	3.5 ± 0.12	17	53
R39	19 ± 4.2	22	60
R41	1.2 ± 0.23	25	84
R42	1.3 ± 0.19	26	95
R43	1.7 ± 0.11	30	89
R44	4.3 ± 0.44	23	65
R46	1.1 ± 0.42	27	94
R48	1.2 ± 0.56	25	76
R50	0.58 ± 0.073	23	84
R35/R39	20 ± 9.4	24	62
Rev_{50-34}[d]	48 ± 7.4	12	46

[a] Affinities were measured at 4 °C by using electrophoretic gel mobility shift assays with radioactively labelled RRE RNA as probe. [b] The amino acid sequence of Rev peptide is Suc-TR(35)QARRN(40)RRRRW(45)R-ERQR(50)RAAAAR-NH_2. [c] Values indicate averages and the standard deviation of at least three experiments. [d] Rev_{50-34} is the reverse sequence of wild-type Rev peptide, AcRQRERWRRRRNRRAQRT-NH_2.

lution structure solved by Williamson et al.,[15] cause larger decreases in binding affinities. For example, the R39, R44 and R38 mutant peptides show a 25-fold, sixfold and fivefold reduction in binding affinity, respectively. Since these three Arg moieties interact with RNA bases through H bonds, methylation likely interrupts the hydrogen-bonding interactions. Compared with the large decreases observed at these three Arg residues, methylation at R35 brings about a 1.2-fold increase in binding affinity. It has been suggested that R35 makes base specific contacts with two different nucleotides.[15] Therefore, the small net increase in binding affinity caused by dimethylation at R35 might be the result of a new hydrophobic interaction that compensates for an interrupted H-bond interaction.

In order to quantify the negative and positive effects that take place in association with changes at major methylation sites, the double mutant peptide R35/R39 (Table 1) was prepared and its binding affinity against RRE was determined. The mutant displayed almost the same binding affinity (K_d = 20 nM) as that of R39; this suggests that the loss in energy caused by the removal of the H bonds is much larger than that gained by introduction of the hydrophobic interaction.

Methylation at all other Arg sites in Rev brought about relatively small changes in binding affinities. While most of these modified proteins have a lower binding affinity, the mutant peptide that is methylated at R50, displayed a 1.3-fold increased binding affinity with RRE (K_d = 0.58 nM). This positive effect on binding can be attributed to the introduction of a new hydrophobic interaction with one base in RRE.[15] The other mutant peptides (R41, R42, R46 and R48) show no change or at best a 1.7-fold decrease in binding affinities relative to wild-type Rev. The Arg residues in these peptides are thought to be involved in minor electrostatic interactions with the negatively charged RNA phosphate backbone.[15] Thus, asymmetric dimethylation causes a minor weakening of these interactions, since it causes the Arg residues to be less basic.[8a] Consequently, if there is a space for asymmetric dimethyl groups on Arg and the distances are appropriate, a new hydrophobic interaction can increase the binding affinity, as seen in the case of R50.[16]

Binding affinities of five selected mutants (R35, R38, R39, R44 and R50) were measured in the presence of $tRNA^{mix}$ and were compared with those values in the absence of $tRNA^{mix}$ as specificity ratios (K_d with $tRNA^{mix}$/K_d without $tRNA^{mix}$; see Table S2 in the Supporting Information). First four mutants were chosen because these positions make the most important contact with RRE; R50 was chosen as the strongest binder. As expected, R35, R38, R39 and R44 showed higher specificity ratios (lower specificities) since the most important hydrogen bonds are absent. Interestingly, the specificity ratio of R50 (1.4) is almost identical with that of the wild type (1.3; Table S2) in spite of new hydrophobic contacts.[21] These data suggest that specificity also depends on the position of Arg methylation.

In order to gain structural information related to the effects of Arg methylation, CD measurements were made with the methylated peptides in the absence and presence of RRE (Table 1). In general, the α-helical contents of Rev proteins are known to increase in the presence of RRE as a result of an induced-fit mechanism.[17,18] Unlike the generally observed reduction of α helicity that is brought about by lysine methylation,[11] the α-helical content of Rev peptides vary according to the position of Arg methylation. Furthermore, a correlation appears to exist between the percentage α helicity and binding affinity (K_a, nM) of the Arg methylated Rev peptides as demonstrated by the plot of data from Table 1 that is shown in Figure 2; this

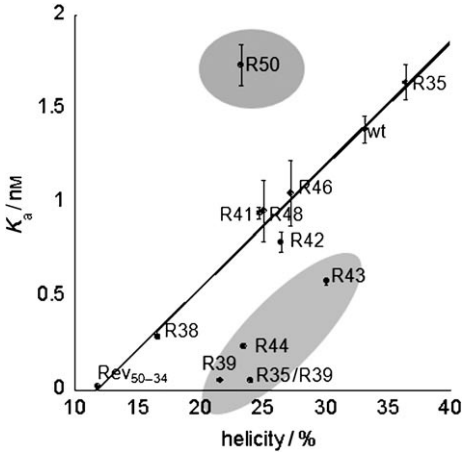

Figure 2. Correlation between α helicity (%) of peptides and association constants, K_a (nM), with RRE RNA; $K_a = -0.75 + 0.064 \times$ helicity, $R = 0.99$.

correlates with the relationship given by Frankel et al.[18,19] Although R35 is at the top of the directly proportional correlation and the reverse sequence reference peptide, Rev_{50-34}, is at the bottom, many exceptions to the correlation exist. Firstly, the R50 mutant has only a moderate α-helical content but it is the strongest binder to RRE. Secondly, the methylated peptides

R39, R43, R44 and R35/R39, in which important hydrogen bonding interactions with RRE are altered, have lower binding affinities than their α-helical contents suggest.

These deviations indicate that α-helical content is one of the factors involved in determining binding affinity. However, other factors appear to govern the effects of Arg methylation on Rev binding to RRE. These include substantial reductions of hydrogen bonding interactions[20] and increases in hydrophobic interactions that are significant enough to compensate for loss of H-bonding interactions. The results of the study described above suggest that the methylation–demethylation process is used as a position specific conformational and interaction switch and that Arg methylation can operate in both a positive and negative manner. It should be noted that positive effects associated with the interaction switch has not been observed in biological system, per se.[11]

Experimental Section

Syntheses of peptides: A library of peptides was synthesized by using the standard Fmoc solid-phase protocol. The N^{α}-Fmoc-N^{ω},N^{ω}-asymmetric dimethylarginine hydrochloride salt was used for asymmetric dimethylarginine synthesis.[14]

Circular dichroism: CD measurements were performed at 20 °C by using a JASCO model J715 spectropolarimeter equipped with Peltier temperature controller and JASCO Spectra Manager™ software. Spectra were acquired from 190–250 nm at scan speed 20 nm min^{-1}, data pitch 0.5 nm, response time 16 s, band width 1.0 nm, and sensitivity 100 mdeg by using 0.1 cm pathlength cuvette, and the signals were averaged over two accumulations.[14]

Electrophoretic gel mobility shift assay: A solution of RRE RNA (10 nM) was heated to 65 °C for 5 min and slowly cooled to room temperature over 1 h in a buffer containing 4-(2-hydroxyethyl)piperazine-1-ethansulfonic acid (HEPES; 20 mM), $MgCl_2$ (1 mM), KCl (5 mM) and NaCl (140 mM, pH 7.4). All samples used for measurements were 10 μL with final concentrations of 1 nM RRE mixed with the indicated amount of peptide in binding buffer (90 mM Tris-borate, 2 mM EDTA, 50 mM $MgCl_2$, 100 mM NaCl, 0.01% Tween® 20). The binding mixtures were incubated on ice for 1 h. Loading buffer (2.6 μL; 50% glycerol in 1.25× Tris-borate and EDTA: TBE buffer) were added to the resulting sample solution. A native polyacrylamide gel (10%; 1 mM $MgCl_2$, 4% glycerol) that had been prerun for 30 min was used. Each sample (12 μL) was loaded on the native polyacrylamide gel (10%) and run at 150 V, 4 °C in 0.5× TBE running buffer (45 mM Tris-borate, 1 mM EDTA) for 2.5–3 h. The gel was exposed to a phosphorimager screen and individual bands were quantified on a FLA-3000 and analyzed with Multi Gauge ver. 3.0 software (Fuji Photo).[14]

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