Two Functionally Distinct Steps Mediate High Affinity Binding of U1A Protein to U1 Hairpin II RNA*

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Phnikoula S. Katsamba‡, David G. Myszka§, and Ite A. Laird-Offringa‡¶

From the ‡Norris Cancer Center/Keck School of Medicine, University of Southern California, Los Angeles, California 90089–9176 and the §Center for Biomolecular Interaction Analysis, University of Utah School of Medicine, Salt Lake City, Utah 84132

Binding of the U1A protein to its RNA target U1 hairpin II has been extensively studied as a model for a high affinity RNA/protein interaction. However, the mechanism and kinetics by which this complex is formed remain largely unknown. Here we use real-time biomolecular interaction analysis to dissect the roles various protein and RNA structural elements play in the formation of the U1A-U1 hairpin II complex. We show that neutralization of positive charges on the protein or increasing the salt concentration slows the association rate, suggesting that electrostatic interactions play an important role in bringing RNA and protein together. In contrast, removal of hydrogen bonding or stacking interactions within the RNA/protein interface, or reducing the size of the RNA loop, dramatically destabilizes the complex, as seen by a strong increase in the dissociation rate. Our data support a binding mechanism consisting of a rapid initial association based on electrostatic interactions and a subsequent locking step based on close-range interactions that occur during the induced fit of RNA and protein. Remarkably, these two steps can be clearly distinguished using U1A mutants containing single amino acid substitutions. Our observations explain the extraordinary affinity of U1A for its target and may suggest a general mechanism for high affinity RNA/protein interactions.

To execute their widely differing functions, RNA-binding proteins must be able to bind to their correct RNA targets with appropriate kinetics, affinities, and specificities (1). In contrast to most DNA-binding proteins, which are presented with a double-stranded B-form helix of uniform structure in which bases can be contacted through the major groove, RNA-binding proteins must be able to bind targets with widely differing structures. Because the steep and narrow groove of double-stranded RNA does not provide proteins easy access to the bases for sequence-specific recognition, most RNA-binding proteins recognize single-stranded regions or distorted double-stranded regions in which the major groove has been widened by bulges, hairpins, or loops (2). The natural variety of RNA targets is bound by a limited collection of RNA-binding motifs (1, 2). The most common of these motifs is the ribonucleoprotein (RNP)1 consensus domain or the RNA-binding domain, also referred to as the RNA recognition motif (RRM). This motif is characterized by two conserved stretches of eight and six amino acid residues (RNP-1 and RNP-2) and a β-α-β-β-α-β secondary structure (see Fig. 1A) (3, 4). RRMs fold into a baseball glove-like structure in which the β-sheet and the surrounding regions form the RNA binding surface. Proteins containing one or more RRMs recognize a variety of RNA sequences and structures (3, 4). An RRM that binds very tightly to its RNA target is the N-terminal RRM of the spliceosomal protein U1A, which binds to an RNA hairpin in the U1 small nucleolar (sn) RNP (U1 hairpin (hp) II or U1hpII) (see Fig. 1).

The U1A/U1hpII interaction has been used as a paradigm for RNA binding by a single RRM and has been the subject of a multitude of biochemical and structural analyses (4). Despite these extensive studies, little is known to date about the mechanism and kinetics of this protein/RNA interaction. Using the previously solved structure of the U1A-U1hpII complex, we have engineered a series of mutants designed to individually examine the roles of electrostatics, hydrogen bonding, aromatic stacking, and RNA loop length, all of which have been implicated in formation of the U1A-U1hpII complex (5–16). The effects of these mutations on the binding dynamics were studied using a surface plasmon resonance-based biosensor (BIA-CORE), which permits the real-time monitoring of complex formation and dissociation (17–19). Our analyses show that complex formation occurs by two clearly distinguishable steps. First, well placed positively charged residues on the protein allow it to rapidly associate with the RNA. Next, close-range interactions at the RNA/protein interface allow the formation of a very stable complex. Together, these steps result in the high affinity of U1A for its U1 hairpin II RNA target (KD ~32 pM). A similar two-step mechanism may play a role in many high affinity RNA/protein interactions.

EXPERIMENTAL PROCEDURES

Construction of the U1A Mutants and Protein Purification—The expression plasmid for the human recombinant U1A protein (amino acids 1–101) was described previously (20). Using this plasmid, a U1A clone with a collection of engineered restriction sites throughout the coding region (U1A-MSHEB) was made by site-directed mutagenesis. All engineered restriction sites were silent at the amino acid level, except a BssHII site that resulted in a Lys88→Arg substitution. Proteins from both plasmids had identical binding properties (data not shown). The MSHEB plasmid was used to generate the mutants used here by digesting the plasmid with the unique restriction sites flanking the amino acid to be mutated and replacing the released fragment with annealed complementary oligonucleotides encoding the specific substitution (in addition to translationally silent restriction sites included for easy identity verification). The mutation in each of the clones was confirmed.

To whom correspondence should be addressed. Tel.: 323-865-0655; Fax: 323-865-0158; E-mail: ilaird@usc.edu.

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‡ To whom correspondence should be addressed. Tel.: 323-865-0655; Fax: 323-865-0158; E-mail: ilaird@usc.edu.

§ The abbreviations used are: RNP, ribonucleoprotein; RRM, RNA recognition motif.
by sequencing and/or restriction digests. All of the clones contained a C-terminally fused MYC tag and a histidine tag used in protein purification. Constructs were transformed into *Escherichia coli* strain BL21/DE3 (Novagen, Madison, WI). Proteins were expressed and purified as described previously (20, 21), with the following modification: a reduced NaCl concentration in the sonication and elution buffers (150 mM NaCl). The active concentration of each protein preparation was determined as described by Christensen (22).

**Gel Shifts—**U1hpII RNA for the gel shift was made as described previously (20), and gel shifts were carried out in a 10-μl final volume of binding buffer (10 mM Tris/HCl, pH 8.0, 150 mM NaCl, 0.5% Triton X-100, 0.25 mg/ml bovine serum albumin, 1 mM dithiothreitol, 0.5 mg/ml tRNA, and 10% glycerol) as described previously (21). Dried gels were analyzed using a Molecular Dynamics PhosphorImager, and bands were quantitated with the ImageQuant software (Amersham Pharmacia Biotech). The *K*<sub>D</sub> value was calculated by plotting the logarithm of the ratio of the complexed/free RNA against the logarithm of the protein concentration (20). The final *K*<sub>D</sub> value given is an average of three independent experiments.

**Biosensor Analysis—**Surface plasmon resonance was used to monitor the interactions of a set of variant U1A proteins binding to a variety of RNA targets under different buffer conditions. Kinetic experiments were performed on both BIACORE 2000 and BIACORE 3000 biosensors (Biacore, Inc., Piscataway, NJ). RNA targets were chemically synthesized (Dharmacon Research, Boulder, CO) with a 5'-biotin tag to allow the capturing of RNA molecules on streptavidin-coated sensor chips. RNA was diluted to 1 μM in HBS buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% surfactant P20) heated at 80 °C for 10 min, cooled to room temperature to allow annealing of the stem, diluted 500-fold in running buffer (10 mM Tris/HCl, pH 8.0, 150 mM NaCl, 5% glycerol, 62.5 μg/ml bovine serum albumin, 15 μg/ml tRNA, 1 mM dithiothreitol, and 0.05% surfactant P20), and injected at 10 μl min<sup>−1</sup>. For U1hpII, 25–35 resonance units of RNA were captured on the streptavidin-coated sensor chip, whereas for the mutant RNAs 100–125 resonance units were captured, as binding to these mutants was significantly weaker, and therefore more RNA was required to generate a reliable binding response. To study the U1A/U1hpII interactions, the proteins were diluted in running buffer and injected at the concentrations indicated in the sensorgrams. In the experiments aimed at determining the effect of the NaCl concentration, the running buffer contained NaCl at 150, 275, 500, and 1000 mM. Binding experiments were carried out at 20 °C and a flow rate of 50 μl min<sup>−1</sup>. Any protein that remained bound after a 5-min dissociation phase was removed by injecting 2 μl NaCl for 60 s at 20 μl min<sup>−1</sup>, which regenerated the RNA surface completely. Analysis of each protein concentration was repeated at least twice, and samples were run in random order. Any background signal from a streptavidin-only reference flow cell was subtracted from every data set. Data were fit to a simple 1:1 Langmuir equation with a correction for mass transport (23) using the global data analysis program CLAMP (24).

**RESULTS AND DISCUSSION**

**Equilibrium Analysis of the U1A/U1hpII Interaction—**The U1A protein, which has a structural role in U1snRNP, has two RRM domains (25). Only the N-terminal RRM domain, however, is required for binding to U1hpII RNA (26–28). The same RRM domain is conserved in all known U1A variants except the human U1A polypeptide using traditional gel shift experiments (Fig. 2A). Triplet experiments yielded an equilibrium binding constant (K<sub>D</sub>) of 4.7 × 10<sup>−11</sup> M<sup>−1</sup>, which agreed well with previously published values (13, 30, 31). Although equilibrium analysis provides information about the affinity of a molecular interaction, it provides no insight into the kinetics underlying the binding mechanism. To obtain kinetic data for the U1A/U1hpII interaction, a BIACORE surface plasmon resonance-based biosensor was used to monitor the formation of the complexes in real time (32, 33).

![Figure 1](http://www.jbc.org/)

**Fig. 1.** U1A protein and U1hpII RNA. A, amino acid sequence of the N-terminal RRM domain (amino acids 1–101) of the human U1A protein. Residues whose interaction with U1hpII were studied are indicated in *boldface*. Residues that were mutated are Lys<sup>50</sup> and Lys<sup>59</sup> (Lys<sup>50A</sup>, Lys<sup>59A</sup>). B, sequence of the U1hpII RNA used for the biosensor analyses. Nucleotides U<sup>5′</sup>–G15 are identical to the wild type sequence. G4 (underlined) was mutated to C in the U1hpII<sup>G4C</sup> variant. The “spacer” nucleotides, whose identity is unimportant for U1A binding, are U8-C10. The molecule is biotinylated at the 5′-end.

![Figure 2](http://www.jbc.org/)

**Fig. 2.** Binding studies of U1A with U1hpII RNA. A, gel shift analysis of U1A with U1hpII. Radiolabeled U1hpII RNA was incubated with increasing concentrations of U1A protein (given in pM below the lanes). Free radiolabeled U1hpII is indicated by *F* whereas *C* represents the shifted complex. The experiment was performed in triplicate. B, BIACORE analysis of the U1A/U1hpII interaction. Biotinylated U1hpII RNA was captured on a streptavidin-coated sensor chip, and increasing concentrations of protein were injected over the surface. The *black lines* represent protein injections performed in triplicate at the indicated concentration. The *red lines* represent the global fit of the entire data set to a single site interaction model including a term for mass transport component. Injections were performed for 60 s followed by 5 min of buffer flow. The kinetic parameters for each of three independent experiments are shown in Table I.

**Kinetics of the U1A/U1hpII Interaction—**To study the kinetics of the U1A/U1hpII interaction on the biosensor, chemically synthesized 5′-biotinylated U1hpII (Fig. 1B) was captured on one BIACORE chip flow cell, whereas a second, unmodified flow cell served as a reference surface. A representative data set for the U1A/U1hpII interaction is shown in Fig. 2B. The
Two Steps in Binding of U1A Protein to U1 Hairpin II RNA

TABLE I

| Mutant Protein | k_d (nM) | k_a (s^-1) | k_d/k_a | KD (nM) |
|---------------|---------|------------|---------|---------|
| Wild type U1A | 4.12    | 0.246      | 25.3    | 6.5     |
| Lys50Ala      | 1.001   | 0.075      | 13.0    | 13.0    |



The kinetics of the U1A/U1hpI interaction are marked by a fast association rate (k_a = 1.1 ± 0.1 × 10^7 s^-1), as well as a slow dissociation rate (k_d = 3.6 ± 1 × 10^-1 s^-1), resulting in a high affinity complex (K_D = 32 ± 7 nM; see Table I). The close agreement of the K_D value obtained using BIACORE with that obtained by gel shift analysis indicates that attachment of the RNA to the BIACORE sensor chip surface does not perturb the reaction thermodynamics. The fast association rate is consistent with the need to include a transport step in the data analysis (23). The association rate surpasses the expected diffusion-based rate constant for two macromolecules in solution (≈10^6 M^-1 s^-1 (34)), suggesting that association may be influenced by electrostatic interactions that increase the odds of productive collisions between the molecules.

Positively Charged Residues Facilitate Rapid Association—To dissect the role of electrostatic interactions in U1A-U1hpII complex formation, we used the U1A-U1hpII co-crystal structure (5) to identify positively charged residues that are located near the RNA binding pocket but are not implicated in hydrogen bonding interactions. Consequently, we examined a number of residues that interact with RNA bases in the splayed-out loop (Arg77 to G11, Arg83 to U3, and Lys88 to C5 (5, 7, 11)). In addition, we avoided mutating Lys and Lys, because the C-terminal region of the RRM had been reported to be required for high affinity binding (13, 14). This left the positively charged residues Lys and Lys, all of which are conserved in U1A from mammals (25, 35), Drosophila (36), Xenopus (X57953), and plants (37) and are also present in the related RNA-binding protein U2B, which binds to a similar stem loop in U2snRNP (25, 38). In the RNA-protein complex, Lys and Lys lie near the base of the RNA stem, in an area between b-strand 1 and a-helix 1 that follows the curve of the double-stranded stem. Lys and Lys could play a role in drawing in the RNA by interacting with the phosphate moiety of nucleotides A–4, U–3, and C–2 (Fig. 3A). Lys lies in the b2-b3 loop region and points into solution in the free protein, whereas in the complex it protrudes through the RNA loop (Fig. 3C). Thus it appears to be well positioned to play a role in attracting the RNA to the binding pocket. To investigate the role of these lysine residues in electrostatic interactions, we replaced them with alanine. Lys and Lys were altered together (Lys20,22Ala mutant), because they appeared to be making similar contacts with the phosphate back-

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The role of electrostatic interactions in the U1A-U1hpII complex.

**A.** U1A-U1hpII complex as seen from the back. The RNA loop is splayed out on the β-sheet surface, which is facing away. Lys20 and Lys22 (indicated in blue) are located in the protein loop connecting β-strands 2 and 3 and protrudes through the RNA loop (purple). **B.** DIACORE analysis of the interaction of Lys20,22Ala with U1hpII (also see the legend for Fig. 2). C. U1A-U1hpII complex seen from the front. Lys50 (indicated in blue) is located in the protein loop connecting β-strands 2 and 3 and protrudes through the RNA loop (purple). **D.** DIACORE analysis of the interaction of Lys50Ala with U1hpII (also see the legend for Fig. 2).

Increasing the NaCl Concentration Reduces the Association Rate—If the roles of the lysine residues are to promote electrostatic interactions with the phosphate backbone, it would be expected that increasing the salt concentration in the buffer would lead to a loss in binding affinity of U1A for U1hpII. Indeed, filter binding experiments showed a hundredfold loss in association rate contributed inordinately to this loss. The Lys50Ala mutation had a minimal effect on dissociation of the complex, indicating that the primary role of this positively charged residue is likely to be in the initial positioning of the RNA loop, possibly through interaction with the exposed phosphate backbone of the free RNA loop. Besides the reduction in its association rate, the Lys20,22Ala mutant also showed an ~4-fold increase in its dissociation rate, indicating that Lys50 and Lys22 also play a moderate role in complex stability. However, for both Lys20,22Ala and Lys50Ala, the major effect on binding resulted from the reduced association rate, indicating the importance of these residues in bringing RNA and protein together.

Increasing the NaCl Concentration Reduces the Association Rate—If the roles of the lysine residues are to promote electrostatic interactions with the phosphate backbone, it would be expected that increasing the salt concentration in the buffer would lead to a loss in binding affinity of U1A for U1hpII. Indeed, filter binding experiments showed a hundredfold loss in U1A-U1hpII equilibrium binding affinity as the NaCl concentration was increased from 150 to 500 mM (31). To assess how the increased NaCl concentration affects the reaction kinetics, we analyzed the U1A/U1hpII interaction at NaCl concentrations of 150 mM, 275 mM, 500 mM, and 1.0 M (Fig. 4). In agreement with results from filter binding assays, we observed a hundredfold increase in the $K_d$ as the NaCl concentration was raised from 150 to 500 mM (Table I). Binding was completely abolished in 1 M NaCl (data not shown). From the analysis of the kinetic data we determined that the loss in affinity was attributable to a decrease in the association rate, which dropped 59-fold as the NaCl concentration was increased to 500 mM. In contrast, the dissociation rate remained relatively constant, varying less than 3-fold across this NaCl concentration range (Table I).

The marked effect of NaCl concentration on the association rate strongly suggests that the initial interaction of U1A with its RNA target is based on electrostatic interactions, which may play a role in prolonging the time the molecules collide, as well as in enhancing the probability of correct alignment (34). If this assumption is correct, mutation of positively charged residues involved purely in electrostatic interactions should diminish the effect of the NaCl concentration. We measured the effect of NaCl concentration on the association rate of the Lys20,22Ala and Lys50Ala mutants and compared them to that obtained for wild type U1A (see Fig. 4D and Table I). The slopes of the log($k_a$) versus log[NaCl] plots were reduced from −3.3 (U1A wild type) to −2.8 for Lys50Ala and −2.4 for Lys20,22Ala. Although both mutants remained sensitive to the salt concentration (which is not unexpected, because the remaining positively charged residues were left intact), the reduction in this effect provides support for a model in which electrostatic interactions play an important role in the rapid association of U1A and U1hpII.

**Aromatic Stacking and Hydrogen Bonding Interactions Stabilize the Complex**—We next examined the kinetic effects of mutations that would prevent stacking or hydrogen bonding interactions that occur in the U1A-U1hpII RNA interface. To this end, the interaction between U1A mutant Phe56Ala and wild type U1hpII and U1hpII mutant G4C (Fig. 1B) and wild type U1A were studied. Phe56 stacks on base A6 in the RNA loop, which in turn stacks on base C7 and Asp92 (Fig. 5A). In the free protein, Phe56 is hidden from the solvent and covered by Ile93. The Phe56:A6 stacking must therefore be accompanied by rearrangements in the protein (12). Base G4 stacks on amino acid Gln54 and also makes hydrogen bond contacts with residues Asn15 and Glu19 (Fig. 5C). Mutation of G4 to C would cause loss of these hydrogen bonds whereas the ability of the base to stack on Gln54 would be maintained. A4 to G mutation had been previously reported to decrease the affinity three to four orders of magnitude (14, 15). Based on previous structural analyses, both the mutant protein and the mutant RNA would be predicted to show strong effects on the dissociation kinetics of the complex, because they are involved in short range interactions that form during the induced fit of RNA and protein. Kinetic analyses of the binding interactions showed that Phe56Ala exhibited a 1400-fold increase in dissociation rate, while showing a less than 5-fold decrease in association rate (see Fig. 5B and Table I). Similarly, the U1hpIIG4C RNA showed a 2500-fold increase in dissociation rate but displayed a less than 4-fold decrease in association rate (see Fig. 5D and Table I). Our observations support the idea that aromatic stacking and hydrogen bonding interactions that mediate the intimate contact of the RNA binding surface and the splayed-out bases do not play a strong role in the initial step of association but are critical for the ability to form a stable complex.

**RNA Loop Size Is Important for Stable Complex Formation**—Several features of U1hpII RNA are critical for recognition, including the presence of a stem, the identity of the closing base pair, and the identity of the first seven of ten loop nucleotides (AUUGCAC) (15, 26, 39). The last three loop nucleotides are thought to function as a spacer and can be replaced by a polyethylene glycol linker without loss of binding affinity (30). Indeed, in the 3′-untranslated region targets, which are very similar in structure and sequence, two of these three nucleotides form part of a stem linking the two targets (40). The need for the spacer nucleotides is linked to the fact that the loop between β-strands 2 and 3 of the protein protrudes through the
RNA loop, where it appears to aid in the splaying of the loop bases so that contacts can be made with the protein β-sheet surface (5). Previous studies of the 10-nucleotide RNA loop had shown that the length of this loop is important for optimal binding (30). Although the identity of the last three loop nucleotides of the RNA target is irrelevant (39), removal of one or more of these nucleotides strongly reduced the binding affinity. We were curious as to how much of this effect was due to the loop may affect the structure of the free RNA in solution and may therefore change the way the RNA is presented to the protein. To distinguish between these possibilities, we analyzed the kinetics of the interaction between U1A and RNAs lacking C9 (U1hpIIΔC9) and U8-C9 (U1hpIIΔUC). Deletion of a single C resulted in a loss of affinity of two orders of magnitude, in accordance with previous equilibrium binding studies (30). Our kinetic analysis demonstrates that this could be attributed almost completely to a 70-fold increase in the dissociation rate of the complex (see Fig. 6A and Table I). The association rate was decreased by less than 4-fold. These data suggest that the role of the three linker nucleotides is indeed that of a spacer, which allows the first seven loop nucleotides to be accommodated on the protein surface. This is supported by the observation that loop nucleotides 8–10 are not visible in the co-crystal due to disorganization (5). Removal of two loop residues had an even more pronounced effect; the $K_d$ increased by over three orders of magnitude (see Fig. 6B and Table I). Again most of this loss in affinity was due to a dramatic increase in the rate of dissociation (~240-fold). Thus we conclude that a minimal
length of the loop is critical to allow assembly of a stable complex. This is consistent with the requirement for the loop to circle the protein β2-β3 loop bulge.

In addition to the dramatic increase in the dissociation rate, a 15-fold loss in the association rate was also seen with the U1hpIIΔUC RNA, suggesting that too much shortening also affects the initial stage of complex formation. Based on NMR studies (11) and molecular dynamics simulations (16), nucleotides 4–10 of the free wild type RNA loop do not appear to be strongly constrained. Perhaps the flexibility of the loop is helpful in establishing initial contacts. This view is supported by the observation that increasing the length of the loop by replacement of U8-C10 with polyethylene glycol linkers two or three times the natural length had a negligible effect on the K_D (30).

**A Multistep Model for Binding**—Our kinetic analyses, combined with structural information about the free and bound proteins and RNA, suggests that formation of the U1A-U1hpII complex proceeds in at least two steps, which we call “lure” and “lock.” First, the protein and RNA are electrostatically attracted through well placed positive charges on the protein and negative charges on the RNA (the phosphate backbone). This initial interaction is followed by a rapid induced-fit event, which locks the RNA and protein into a stable complex. The presence of positively charged residues surrounding the RNA binding pocket supports this notion. These positive charges could aid association by increasing the time that the free RNA and protein remain close together following a random collision, thereby increasing the odds that during subsequent collisions, both molecules will adopt an orientation compatible with locking (34, 41). In this scenario, flexibility of the free RNA loop would facilitate establishment of the initial electrostatic contacts, allowing the RNA backbone to “mold” onto the RNA binding site. As soon as the orientations of the RNA and protein are compatible, close-range interactions could initiate between the two molecules, resulting in interactions that require rearrangements in protein and RNA (such as stacking of Phe on A6). An induced-fit mechanism in which the RNA, the protein, or both adapt during complex formation appears to play a role in many RNA/protein interactions (42). Our observation that this induced fit (lock) is preceded by an electrostatically-mediated binding step (lure) warrants detailed kinetic investigations of other RNA/protein complexes. The distribution of positively charged residues along the RNA binding tract of poly(A) binding protein (43), Sex-1 (44), and nucleolin (45), three multi-RRM proteins, suggests these proteins bind RNA by a similar two-step mechanism. The initial electrostatically based association step may offer a way of engineering RNA-binding proteins with increased affinity for their targets, through the introduction of more positively charged residues near the RNA binding area, leading to an increase in the association rate.

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