METHOD

Quantitation of free energy profiles in RNA–ligand interactions by nucleotide analog interference mapping

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ABSTRACT

RNA interactions with protein and small molecule ligands serve a wide variety of biochemical functions in the cell. To best understand the specificity and affinity of these interactions, the free energy contribution made by individual function groups in the RNA must be determined. As an efficient method for obtaining such energetic profiles, we report quantitative nucleotide analog interference mapping (QNAIM). This extension of the NAIM methodology uses the magnitude of analog interference as a function of ligand concentration to calculate binding constants for RNA with individual analog substitutions. In this way, QNAIM not only defines which functional groups are important to an interaction but simultaneously determines the energetic contribution made by each occurrence of that functional group within the RNA polymer. To establish the utility of this approach, QNAIM was used to quantify functional group interactions within the signal recognition particle (SRP), specifically the 4.5S RNA with the M domain of Ffh. In each of the cases in which energetic data were available from previous site-specific substitution analyses, QNAIM provided nearly equivalent results. These experiments on a model system demonstrate that QNAIM is an efficient method to establish a chemically detailed free energy profile for a wide variety of RNA–ligand interactions.

Keywords: SRP; RNA–protein interaction; NAIM; energetic analysis; binding constants

INTRODUCTION

RNA–protein particles play critical roles in many cellular processes (Cusack 1999; Hall 2002). The structure of these complexes is maintained through an array of interactions between the RNA and the protein, including hydrogen bonding, electrostatic, and van der Waals interactions. This collection of interactions defines the specificity and affinity of the RNA for its ligand.

The energetic contributions made by individual functional groups at the interface of an RNA–protein interaction have been determined for only a small number of RNA–protein complexes (Draper 1999); yet, it is in these interactions that the free energy of protein–RNA binding is defined. The reason such data are rarely reported owes to its difficulty of collection. Much of the work that has been done to define the energetics of RNA–protein binding in-

terfaces has relied on traditional mutagenesis (Witherell et al. 1991; Hall 1994; Conn et al. 1998; Xu et al. 2002). Such substitutions often have more global effects on RNA structure, which makes them a crude tool for dissecting the contributions to the binding energy of an RNA–protein particle (Nameki et al. 1999; Shen et al. 1999; Kim and Tinoco 2001). In addition, mutagenesis only affects bases and is not useful for examining contributions made by the ribose backbone. Alternatively, synthetic RNAs containing site-specific analog substitutions have been used to probe contributions from individual functional groups (Carey et al. 1983; Baidya and Uhlenbeck 1995; Elliott et al. 1999; Pleiss and Uhlenbeck 2001). Such modifications are often difficult and expensive to prepare, particularly on large RNAs. Thus, although x-ray crystal structures of RNA–ligand complexes suggest which interactions are responsible for the affinity of the complex (Oubridge et al. 1994; Valegard et al. 1994; Antson et al. 1999), high-resolution biochemical experiments are infrequently performed to confirm or refute these energetic predictions.

To expedite the collection of such data we have extended nucleotide analog interference mapping (NAIM) to provide not just a qualitative but a quantitative assessment of an RNA–ligand interaction at functional group resolution. NAIM is a well-established technique used to determine the

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chemical basis of RNA structure and function (Strobel 1999). In a single NAIM experiment, the effect of modifying a particular base or sugar can be simultaneously, yet individually, tested at every position within an RNA molecule. An extensive series of phosphorothioate-tagged nucleotide analog triphosphates are now commercially available for

random enzymatic incorporation into an RNA transcript (Glen Research Inc.). After a physical or biochemical selection, the positions of analog incorporation that are detrimental to activity can be determined by breaking the phosphorothioate tag with I_2 and resolving the cleavage products on a denaturing gel. Positions at which a particular substitution is detrimental to RNA function

are displayed as absent or less intense bands on the sequencing gel. The magnitude of the interference provides some indication of the relative energetic or kinetic penalty paid by a particular modification. Although NAIM interference provides a qualitative assessment of which functional groups contribute to binding, it is not a quantitative measure of that contribution. To address this issue, we have developed quantitative NAIM (QNAIM).

QNAIM is a series of NAIM experiments performed at several ligand concentrations (Fig. 1). The RNA concentration is held substantially below the dissociation constant of the complex (K_d), and the ligand concentration is varied from one to several times the K_d concentration. Under these conditions, RNA–ligand binding is driven by the increase in the ligand concentration, which is already in vast excess. As a result, the RNAs do not compete with each other for binding; instead, the distribution of RNAs in the bound fraction results entirely from the intrinsic affinity of each RNA for the ligand. The population distribution in the bound fraction is revealed by the individual band intensities observed in the sequencing ladder that results from iodine treatment. By plotting the interference magnitude as a function of ligand concentration, it is possible to calculate the binding constant and free energy contribution ($\Delta\Delta G$) of each functional group substitution at each position in the RNA.

As a model system to test the utility of QNAIM for quantifying the free energy contribution of individual function groups within an RNA ligand interaction, we selected the complex formed between the 4.5S RNA and the M domain of the Ffh protein (Struck et al. 1988; Poritz et al. 1990). This interaction constitutes the core of the *Escherichia coli* signal recognition particle

FIGURE 1. Schematic diagram of a QNAIM experiment. In the first step, labeled RNAs with randomly incorporated phosphorothioate-tagged analog nucleotides are incubated with concentrations of protein ranging from two- to 500-fold above the K_d and are allowed to reach equilibrium. The bound and unbound RNA fractions are physically separated by filter binding, gel electrophoresis, or some other method. Because the protein is in excess, RNA binding is noncompetitive, and the fraction of any given RNA species bound is due to the intrinsic affinity of the RNA for the protein. In this example, the analog at the second position affects binding, causing only a small fraction of the RNA to be bound at low protein concentrations. The bound fraction is cleaved with iodine, and the products are separated by polyacrylamide gel electrophoresis to reveal the position at which the incorporation of a nucleotide analog has altered the binding constant of the RNA for the protein. The relative intensity of each band at each ligand concentration is then used to calculate the binding constant and the free energy cost resulting from functional group substitution at each position in the RNA.

RESULTS AND DISCUSSION

The analog 2'-deoxyguanosine (dGαS) provided an example of how two positions can be simultaneously, but independently, analyzed in a single experiment. 2'-Deoxy-

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FIGURE 3. A representative QNAIM data set obtained by using the analog dA α S. (A) Interference pattern from A α S incorporation. (B) Interference pattern from dA α S incorporation. Protein concentration ranges from one to 1024 times the K_d of the bulk RNA, which in this case was from 30 to 30720 pM. (C) Plot of interference magnitude ($1/\kappa$) versus protein concentration for dA α 39 interference.

guanosine causes interference at two positions within the RNA: G49 and G61 (Fig. 4B). When analyzed by QNAIM, these interferences were rescued at different protein concentrations, which reflect differences in their binding constants. At position G49, the dG α S substitution gave a threefold increase in K_d , corresponding to a loss of ~ 0.5 kcal/

mole in binding energy; whereas at position G61 the substitution resulted in a fivefold increase in K_d , revealing a 0.8 kcal/mole loss in binding energy.

For those functional groups with substitution that is particularly deleterious, substituted RNAs were not retained on the filter and therefore did not show rescue even at the

TABLE 1. Binding energies of mutants determined by QNAIM and those previously reported by site-specific substitution analysis (Batey et al. 2000)

Nucleotide position	Change in free energy (kcal/mole) determined by QNAIM	Change in free energy (kcal/mole) determined by site-specific mutagenesis
dA39	1.7 ± 0.4	1.8 ± 0.14
dC40	0.97 ± 0.3	
Pur47	>3.3	2.3 ± 0.15
I48	0.86 ± 0.4	0.61 ± 0.16
dG49	0.50 ± 0.3	0.91 ± 0.16
dG61	0.81 ± 0.1	1.1 ± 0.18
dC62	>4.4	5.2 ± 0.5

QNAIM errors are based upon variation observed in three independent experimental measurements.

highest protein concentrations. This was evident in two cases: purine substitution at A47 and a 2'-deoxycytidine at C62. Again, this is consistent with the site-specific substitution analysis. Retention of the dC62-substituted RNA was so inefficient that a competition assay was necessary to determine the binding constant of this RNA. Although it was not possible to measure a binding constant at this position, it was still possible to define a minimum limit of 4.2 kcal/mole for the energetic penalty resulting from deletion of this functional group. This is of a magnitude consistent with the measured value of 5.1 kcal/mole. When purine is incorporated at position 47, the low efficiency of retention, even at high protein concentrations, made it possible only to establish a lower estimate of the effect. This estimate is ~ 1 kcal/mole greater than that measured by site-specific substitution. However, in the previous work, the fraction of RNA bound was $<20\%$, and even this small fraction bound was achieved only when the filters were not washed. This is

a fraction too small to detect in the present assay, and the filters had to be washed to avoid excessive background, which may explain the difference between these two values.

Although M-domain binding to the 4.5S RNA provided an ideal system for establishing the reliability of the methodology, because of the availability of functional group specific binding data, this system presented some challenges for this type of analysis. The binding constant for the interaction is quite low, at 35 pM (Batey et al. 2001). To accurately measure the K_d , the RNA concentration was held ~ 10 -fold below the binding constant (3 pM). Although it is straightforward to carry out a filter binding experiment with a uniform RNA population at 3 pM RNA, producing sufficient RNA in the binding experiment to subsequently visualize on a sequencing gel introduced some technical challenges. Initially, various concentrations of $MgCl_2$ and KCl were used in the binding buffers to affect the binding constant of the complex. These studies were successful, and the

FIGURE 4. (A) Plot of interference magnitude versus protein concentration for several other interferences within the 4.5S RNA: dC41 (circles), I48 (squares), dG49 (diamonds), and dG61 (crosses). (B) QNAIM data obtained by using the analog dGαS.

binding constant was raised to the much more convenient value of 1 nM. However, at high Ffh concentrations, we observed increased nonspecific binding of the RNA to the protein. At an Ffh concentration of >100 nM protein, the RNA began to bind to the nitrocellulose filter nonspecifically in what appeared to be precipitated aggregates. Therefore, the experiments had to be carried out under the higher salt concentrations that afforded a binding constant of 30 to 40 pM. Because the RNA concentration was so low, at least a dozen dot blots had to be collected for every protein concentration in order to yield enough RNA for individual band detection on the interference gel. At the lowest protein concentrations, in which the fraction bound was <20%, >12 dot blots were collected for each protein concentration. Although inconvenient, such challenges were manageable and are unlikely to be common to most systems.

Although QNAIM provides efficient thermodynamic analysis of an RNA–protein interaction, there are some limitations to this approach that should be noted. Any chemical alteration in an RNA molecule can induce both local and global effects (Nameki et al. 1999; Shen et al. 1999; Kim and Tinoco 2001). These perturbations may lead to measured changes in binding affinity that encompass more than simply the loss of a hydrogen bonding interaction resulting from substitution of a single functional group. Thus, although QNAIM quantifies the magnitude of the effect, it does not specify the cause of the energetic penalty. Such complexities of interpretation are not unique to QNAIM but arise in any experiment in which nucleotides have been mutated or modified to measure changes in binding energy.

To explore the cooperative nature of effects within a thermodynamic profile, it is helpful to perform an analysis in which two or more positions are substituted. Such a study is not possible using a QNAIM experiment as outlined here. However, some variations could be implemented if such data are desired. Once key nucleotides have been identified, QNAIM analysis could be repeated in the context of a point mutation to see how the energetic profile is affected. Alternatively, if chimeric RNAs with site-specific substitutions and randomly incorporated phosphorothioate-tagged molecules are prepared, then QNAIM could be used to identify cooperative effects between particular substitutions.

These results on a model system establish QNAIM as an efficient method to establish a chemically detailed free energy profile for a variety of RNA–ligand interactions. The 4.5S RNA contacts the Ffh protein through several key functional groups, all of which were identified by using traditional NAIM techniques (Batey et al. 2000). By performing the analysis as a function of protein concentration, the energetic contribution made by each of the functional groups could be quantified. RNAs provide the scaffold, and in some cases the catalytic residues, for many key structures in biological function, but they are rarely found in the absence of proteins (Cusack 1999; Draper 1999; Hall 2002). Although

analysis of protein–RNA complexes is one application for this technique, it could also be used to measure binding energies in RNA–RNA interactions and RNA–small molecule interactions. In addition, the existence of a large catalog of modified nucleotide phosphorothioate triphosphates means that a number of functional group substitutions, which were previously untestable, can now be examined for their role in protein–ligand interactions. QNAIM will make it more convenient to elucidate the energetic basis of these RNA–ligand interactions in chemical detail.

MATERIALS AND METHODS

Materials

Plasmid pUC194.5S was used for RNA expression (Batey et al. 2001). The Ffh (328–432, C406S) M-domain protein was expressed and purified as previously published (Batey et al. 2001). Nucleotide analog 5′-O-(1-thio) triphosphates were synthesized as described (Ryder and Strobel 1999) or purchased from Glen Research Inc.

Methods

The 4.5S RNA was prepared for use in the QNAIM experiments by *in vitro* run-off transcription. Plasmid pUC194.5S encoding the 4.5S(1–110) RNA was linearized by using *Bam*HI and transcribed for 4 h at 37°C in a reaction containing 40 mM Tris-HCl (pH 7.5), 1 mM spermidine, 5 mM dithiothreitol (DTT), 0.01% Triton-X-100, 15 mM MgCl₂, 1 mM each 5′ nucleotide triphosphate, 20 μg/mL linearized DNA, and 1 μg/mL T7 RNA polymerase. The reaction mixture also contained one of several different 5′-O-(1-thio) nucleoside analog triphosphates at a concentration necessary to produce ~5% incorporation (an average of slightly less than one per molecule for a transcript of this size; Ryder and Strobel 1999). The analogs included either parent nucleosides, in which the only substitution was replacement of the nonbridging phosphate oxygen with sulfur, or nucleoside analogs that contained the sulfur substitution and a second chemical alteration. For those transcripts containing 2′-deoxy-5′-O-(1-thio) nucleosides, the mutant Y639F T7 RNA polymerase was used (Sousa and Padilla 1995; Padilla and Sousa 1999). The RNA construct contained a 5′ hammerhead ribozyme and a 3′ hepatitis delta virus ribozyme to ensure transcripts of uniform length. To promote cleavage by both ribozymes, the transcription reactions were heated for 10 min at 70°C and slow-cooled to room temperature. The cleaved RNA was gel-purified on an 8% polyacrylamide/7 M urea denaturing gel, visualized by UV shadowing, and physically cut from the gel. The RNA was eluted in 2% SDS and T₁₀E_{0.1} (pH 7.5) for at least 2 h at room temperature, phenol-chloroform extracted, and precipitated by the addition of three volumes of ethanol and freezing. The RNA was then resuspended in 100 μL of T₁₀E_{0.1} (pH 7.5). A small amount of the RNA was labeled by using T4 polynucleotide kinase (Roche) and γ-³²P-ATP (NEN). The labeled RNA was then gel-purified, eluted, precipitated, and stored in T₁₀E_{0.1} (pH 7.5).

Experimental overview

The QNAIM experiment included the following steps: The RNA was incubated with several different protein concentrations for a

time sufficient to reach equilibrium. The binding constant for the population of RNA was determined by using a filter binding assay. The RNA–protein sample at each concentration was applied to a nitrocellulose filter to separate the bound from the free RNA. The bound RNA fractions at each protein concentration were recovered from the filter by incubation in elution buffer (300 mM NaOAc at pH 5.3, 1% SDS, 1 mM EDTA). These RNA fractions were then cleaved with iodine, and the products were resolved on a polyacrylamide gel. The intensities of the individual bands were quantitated and used to calculate the change in binding constants for analog substitution at each position. Experiments were carried out in an equivalent manner for each analog tested.

Filter binding analysis

The macroscopic binding constant for the entire RNA population for each transcription was determined by filter binding analysis (Wong and Lohman 1993). A constant concentration (~5 pM) of the 5′-end-labeled RNA was heated for 1 min at 90°C and cooled on ice for at least 3 min. The RNA was incubated with concentrations of protein ranging from 10 to 25,000 pM in 20 mM Tris-HEPES (pH 7.5), 200 mM KCl, 10 mM MgCl₂, 1 mM DTT, 0.01% Igepal C-680, 0.025 mg/mL tRNA, and 0.5 mM Na₂EDTA (pH 8.0) for at least 1 h. Two filters, a BA85 nitrocellulose filter (S&S) and a positively charged nylon Hybond N+ filter (Amersham Pharmacia), were soaked in wash buffer (20 mM Tris-HEPES at pH 7.5, 200 mM KCl, and 10 mM MgCl₂) for 1 h and placed in a dot blot apparatus. The nitrocellulose filter was placed on top to bind the protein and any associated RNA, whereas the underlying nylon filter retained the unbound RNA. A 40 μL aliquot of each protein-RNA mixture was individually applied to the filters and rinsed with 100 μL of wash buffer. Both the nitrocellulose and Hybond-N+ filters were air dried and exposed to PhosphorImager plates, and the amount of RNA bound to each of the filters was quantified by using ImageQuant (Molecular Dynamics). The macroscopic binding constants were calculated by using a least-squares fit to the following equation:

$$\text{fraction bound} = \frac{(M * [\text{protein}])}{(K_d \text{ bulk} + [\text{protein}])} + L \quad (1)$$

where K_d bulk is the apparent equilibrium binding constant of the entire RNA population; M , which varies from 0.7 to 0.9, is the maximum fraction of RNA bound at the highest protein concentration; and L , which is usually very close to zero, is the minimum fraction of RNA bound at the lowest protein concentration.

QNAIM

At least twelve 40 μL aliquots of each RNA–protein mixture at every protein concentration were applied to 12 dots on the filters, and each was rinsed with 100 μL of wash buffer. The individual dots on the nitrocellulose filter for a given protein concentration were collected and placed into a single tube to which 700 μL of elution buffer (300 mM NaOAc at pH 5.2, 1.0% SDS, 1 mM EDTA) was added. The tubes were rocked for 1 h, and the eluted RNA was ethanol precipitated and resuspended in loading buffer (95% formamide, 0.025% bromophenol blue, 0.025% xylene cyanol, 25 mM Na₂EDTA). For every protein concentration, two

samples were prepared. To one, 1 μL of 100 mM I₂ was added to cleave the phosphorothioate linkages. Nothing was added to the second sample, which served as the control for background degradation in the RNA. The samples were heated for 3 min at 90°C, and cleavage products were resolved on an 8% polyacrylamide/7 M urea gel. In addition to controlling for the background level of degradation, controls for the level of incorporation were run. Unselected 5′-end-labeled RNA was cleaved, and the cleavage products were resolved on an 8% polyacrylamide/7 M urea gel. The gels were exposed to PhosphorImager plates, and the individual band intensities were analyzed by using ImageQuant (Molecular Dynamics).

Interference band analysis

Peak intensities for both the parental nucleotide (NαS) and the nucleotide analog (δαS) were quantitated by PhosphorImager analysis at each position in the RNA sequence for both the bound RNA fraction and the unselected 5′-end-labeled control. Band intensities were measured by using gel-area integration and were baseline-corrected to a blank region of the gel. Interference (1/κ) values for each nucleotide position were calculated by using the following formula:

$$1/\kappa = NF \times \frac{(\delta\alpha S \text{ experiment}/N\alpha S \text{ experiment})}{(\delta\alpha S \text{ 5' control}/N\alpha S \text{ 5' control})} \quad (2)$$

where NF represents the cumulative normalization factor necessary to correct for loading differences and variability in the extent of the retention of the RNA on the filter. NF is typically between 0.8 and 1.2 and was determined by measuring the intensities of at least six bands unaffected by protein concentration and averaging the value.

The 1/κ value was used to determine the binding constant for each position of interference. The data were fit to the equation

$$1/\kappa = \frac{(M * [\text{protein}])}{(K_d \text{ interference} + [\text{protein}])} + L \quad (3)$$

where K_d interference is the apparent equilibrium binding constant of the RNA population with a substitution at a particular site; M is the maximum level of rescue of the particular interference (or the upper baseline) and L is the maximum extent of interference for the same position (or the lower baseline). The change in binding free energy was computed by using the measured bulk binding constant and the binding constant determined for the particular interference band using the equation:

$$\Delta\Delta G = -RT * \ln(K_d \text{ bulk}/K_d \text{ interference}) \quad (4)$$

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