A New Method To Detect Long-Range Protein−RNA Contacts: NMR Detection of Electron−Proton Relaxation Induced by Nitroxide Spin-Labeled RNA

Andres Ramos and Gabriele Varani*

MRC Laboratory of Molecular Biology
Hills Road, Cambridge CB2 2QH, England

Received July 15, 1998
Revised Manuscript Received September 10, 1998

Protein−RNA complexes play a central role in many biochemical processes, and studies of RNA−protein recognition are critical to understand how gene expression is regulated.[1] However, only a few structures of RNA−protein complexes have so far been determined at atomic resolution.[2,3] The relatively large molecular weight of such complexes represents a serious limitation for NMR studies. Structures of protein−nucleic acid complexes are determined by extracting NOE-derived intermolecular distance constraints. This method works well for systems of molecular weights of 40 kDa or less, characterized by tight binding ($K_d \approx 10^{-9}$ M), highly specific recognition. However, the sensitivity of heteronuclear edited/filtered NOESY experiments[4] decreases significantly as the molecular weight increases. Furthermore, many proteins of great biochemical interest bind RNA rather weakly ($K_d \approx 10^{-6}$ M) and with poor specificity. When these conditions occur, intermolecular NOE interactions can be quenched by dynamic processes at the interface, making high-resolution structure determination very difficult. In this communication, we describe a method to overcome these limitations. Our approach is based on intermolecular electron−proton dipolar relaxation and allows the extraction of long-range intermolecular distance information in protein−RNA and protein−DNA complexes.

Paramagnetic spin labels have a long history in NMR spectroscopy, and have lately been rediscovered in studies of protein-folding [5,6] and DNA-drug complexes.[7] The paramagnetic species increase the relaxation rate of NMR resonances in the vicinity of the unpaired electrons, in proportion to the inverse sixth power of the distance between the label and the reporter nucleus. To apply this principle to the study of RNA−protein complexes, we adapted spin labeling techniques to RNA chemistry and detected electron−proton relaxation by observing changes in intensity and line shape of protein resonances in the complex by recording heteronuclear correlated spectra.

The system we wanted to study is the complex between the third double-stranded RNA-binding domain of the *Staufen* protein from *Drosophila* (dsRBD3)[8] and double-stranded RNA (Figure 1). Double-stranded RNA-binding domains are ubiquitous modules[9] that recognize double-stranded RNA of any sequence. 1/2X filtered NOESY spectra utilizing either labeled protein or labeled RNA yielded few intermolecular distance constraints. The great majority of observed intermolecular NOE interactions involve RNA backbone resonances (H4′, H5′, and H5″ protons); these are very difficult to assign to specific residues due to spectral overlap, even with labeled RNA.

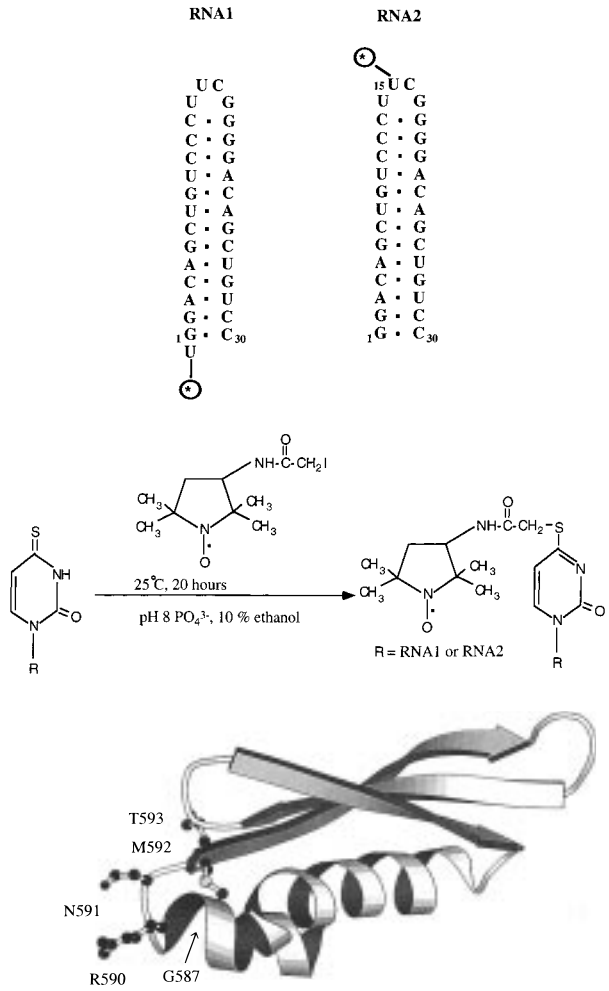

Figure 1. (a) Sequence and secondary structure of the RNAs used in this study; the sites of labeling in each of two preparations are indicated by a star. (b) Scheme of the coupling reaction. The crude RNA was purified by gel electrophoreses, electroeluted, ethanol precipitated, then dissolved in 100 mM phosphate buffer at pH 8. 3-(2-Iodoacetamido)-proxyl was dissolved in water/ethanol (70% v/v) and added to the RNA in 100-fold excess. The reaction mix was incubated for approximately 20 h at room temperature in the dark under vigorous shaking.[10] Progress of the reaction was monitored by UV spectroscopy and confirmed by NMR. After activation, the labeled RNA was purified by passage through a NAP-10 size exclusion column, ethanol precipitated, and resuspended in the NMR buffer (10 mM Na-phosphate, pH 6.5). Less than 20% of the RNA was lost during coupling and purification. (c) Molscript representation of the structure of *Drosophila Staufen* dsRBD3 protein;[8] amino acids in close proximity with the spin-label on RNA-1 are labeled.

Proxyl spin labels (Figure 1b) were attached to chemically synthesized RNA containing single 4-thio uracyl bases at the desired positions. After purification of the RNA by gel electrophoreses, 3-(2-iodoacetamidoproxyl) was coupled to 4-Thiouracyl by standard chemistry [10] (Figure 1b). Progression of the coupling reaction was followed by UV spectroscopy. The maximum in UV absorption for 4-Thiouracyl is shifted to ≈320 nM; this peak disappears when coupling occurs. The reaction was essentially complete overnight at room temperature. No degradation of the

* Address correspondence to this author: gv1@mrc-lmb.cam.ac.uk.

(1) Varani, G. *Acc. Chem. Res.* **1997**, *30*, 189−195.
(2) Nagai, K. *Curr. Opin. Struct. Biol.* **1996**, *6*, 53−61.
(3) Varani, G.; Nagai, K. *Annu. Rev. Biophys. Biomol. Struct.* **1998**, *27*, 407−445.
(4) Otting, G.; Wüthrich, K. *Q. Rev. Biophys.* **1990**, *23*, 39−96.
(5) Gillespie, J. R.; Shortle, D. *J. Mol. Biol.* **1997**, *268*, 158−169.
(6) Gillespie, J. R.; Shortle, D. *J. Mol. Biol.* **1997**, *268*, 170−184.
(7) Dunham, S. U.; Dunham, S. U.; Turner, C. J.; Lippard, S. J. *J. Am. Chem. Soc.* **1998**, *120*, 5395−5406.
(8) Bycroft, M.; Grünert, S.; Murzin, A. G.; Proctor, M.; St Johnston, D. *EMBO J.* **1995**, *14*, 3563−3571.
(9) St Johnston, D.; Brown, N. H.; Gall, J. G.; Jantsch, M. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 10979−10983.
(10) Coleman, R. S.; Kesicki, E. A. *J. Am. Chem. Soc.* **1994**, *116*, 11636−11642.

10.1021/ja982496e CCC: $15.00
Published on Web 10/07/1998

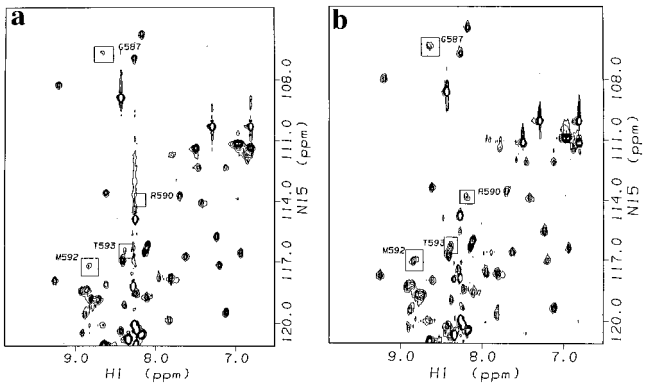

Figure 2. Section of the $^{1}H-^{15}N$ HSQC spectra of ^{15}N-labeled *Staufen* dsRBD3 protein and spin-labeled RNA-1 recorded before (a) or after (b) reduction of the spin label by adding 5 mM sodium hydrosulfite. Protein−RNA complexes were prepared by mixing pre-dyalized protein and RNA in equimolar amounts at a concentration of 0.05 mM (RNA-1) or 0.2 mM (RNA-2; data not shown). HSQC experiments [13] were recorded at 300 K on a Bruker DMX-600 spectrometer in ≈20 h.

RNA was observed during the reaction or the subsequent purification of the spin-labeled RNA.

The choice of site of labeling is crucial; this must be outside the protein binding site to minimize any perturbation of the intermolecular interface, yet sufficiently close to allow the observation of intermolecular paramagnetic relaxation. Spin labels were inserted at two positions in different RNA samples (Figure 1a). Both sites were close to but do not overlap with the expected binding site. The sites of modification were also chosen to coincide with nucleotides not involved in the stabilization of RNA secondary structure. NMR data confirm that the RNA structure is unaffected by the modification (not shown). Furthermore, $^{1}H-^{15}N$ HSQC spectra recorded either with unmodified RNA samples or with spin-labeled RNAs were very similar. Thus, attachment of the nitroxide spin labels did not significantly perturb the structure of the protein−RNA complex.

Heteronuclear $^{1}H-^{15}N$ HSQC spectra of the complex between spin-labeled (Figure 2a) or reduced (Figure 2b) RNA-1 and *Staufen* dsRBD3 protein have been recorded under identical conditions. Some resonances in the protein are broadened by the proxyl label (compare cross-peaks marked with residue labels in Figure 2a and 2b; notice that some peaks disappear from the spectrum altogether). The location of these residues is mapped on the protein structure in Figure 1c. The range of the paramagnetic relaxation effect was semiquantitatively estimated by observing by 1D-spectroscopy RNA imino resonances that provided an internal control. Since the RNA structure in the complex has been determined in the course of this study (unpublished), we can estimate that resonances within ≈12 Å of the site of attachment are broadened beyond detection. The line width of resonances within ≈16 Å increases significantly, but the corresponding peaks remain observable in 1D ^{1}H spectra. We have been able to collect 12 long-range distance constraints (up to approximately 15 Å) between the RNA and the protein from two test samples, using only ^{15}N-labeled protein and very small amounts of sample (concentrations as low as 0.05 mM were used). Increasing the concentration would allow a quantitative estimate of the paramagnetic contribution to the relaxation times T1 and T2,[6,7] and this would in turn allow the extraction of many more, possibly longer range constraints.

The results presented here introduce a new approach to obtaining intermolecular long-range distance constraints in protein−RNA and protein−DNA complexes. The sensitivity of the method is very high and this approach should remain effective at very high molecular weight by using random fractional deuterated samples and line-narrowing techniques.[11] This approach can provide long-range information for systems that can be studied with NOE-based methods. The results presented here demonstrate that it can also provide intermolecular distance constraints in cases where NOE interactions are unobservable or cannot be assigned. A strategy for the determination of structures where weak and/or poorly specific binding limits the NOE-based approach would use qualitative estimates of proton−electron distances to dock predetermined protein and RNA structures and obtain first models for the complex. These structures would allow the identification of ambiguous NOE interactions and would provide quantitative estimates of electron−proton relaxation rates during the refinement of the structure, as demonstrated in studies of paramagnetic proteins.[12]

Acknowledgment. It is a pleasure to thank Mr. Richard Grenfell for RNA synthesis and purification; Drs. Mark Farrow, Mike Gait, and Tariq Rana (Rutgers University) for advice; Dr. Mark Bycroft, Mr. Mark Proctor (Cambridge Center for Protein Engineering), and Dr. Stefan Grunert (IMP, Vienna) for purified Staufen dsRBD3 protein; and Dr. Gerhard Wagner (Harvard Medical School) for bringing the opportunities provided by this method to our attention. A.R. acknowledges the European Union for a training fellowship.

JA982496E

(11) Pervushin, K.; Riek, R.; Wider, G.; Wüthrich, K. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 12366−12371.

(12) Bertini, I.; Donaire, A.; Luchinat, C.; Rosato, A. *Proteins* **1997**, *29*, 348−358.

(13) Mori, S.; Abeygunawardana, C.; O'Neil Johnson, M.; van Zijl, P. C. M. *J. Magn. Reson.* **1995**, *B 108*, 94−98.