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^{1}H, ^{13}C, ^{15}N resonance assignment of the 20 kDa double stranded RNA binding domain of PKR

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Biological context

The interferon (IFN)-induced double-stranded RNA (dsRNA)-activated protein kinase PKR plays a key role in the antiviral and antiproliferative activities of IFN (see reviews, Williams, 1995; Clemens, 1997). The antiviral activity of PKR is mediated through the phosphorylation of initiation factor eIF-2α and subsequent inhibition of protein synthesis. More recently, PKR has been shown to activate several transcription factors including NFκB and IRF-1 in response to specific extracellular stimuli, which has led to the suggestion that PKR is involved in normal control of cell growth and differentiation and acts as a signal transducer at both the transcriptional and translational levels (Williams, 1995; Clemens, 1997). A unique and critical step in the activation of PKR, compared to other kinases, is the sequence-independent dsRNA interaction with its N-terminal RNA binding domain. Upon binding to dsRNA, PKR is thought to undergo conformational rearrangement and autophosphorylation, which leads to the phosphorylation of eIF2 and other target proteins. The dsRNA binding domain of PKR comprises two tandem copies of a 65–68 amino acid dsRNA binding motif (dsRBM), which has been identified in dozens of functionally diverse RNA binding proteins (Kharrat et al., 1995). Both dsRBM repeats in PKR are required for its cooperative and high affinity binding to dsRNA, as shown by quantitative gel mobility-shift and filter-binding assays (Schmedt et al., 1995; Bevilacqua and Cech, 1996). To gain insight into the molecular mechanism of PKR activation by dsRNA, we have initiated NMR structural studies of the dsRNA binding domain (dsRBD) of PKR and its interaction with the dsRNA. Herein, we report the resonance assignments of dsRBD (20 kDa) using multidimensional heteronuclear NMR spectroscopy. Although the resonance assignment of small or medium sized proteins can be readily obtained using 3D and 4D heteronuclear NMR experiments, the resonance assignment of proteins or domains in the 20–30 kDa range is still a major step towards their final structure determination. In the case of dsRBD, which contains two repeated motifs, there are repeated residues in the sequence such as leucines and lysines, which cause chemical shift degeneracy for the backbone amides or side-chains. By using a series of two-, three-, and four-dimensional through-bond and through-space heteronuclear NMR experiments, we have been able to obtain nearly complete assignments for both backbone and side-chains.

Methods and results

E. coli BL21(DE3)plysS cell culture (4 L) transformed with the vector pET-15b (Novagen, Inc) encoding dsRBD of human PKR was grown at 37 °C and induced with 1 mM IPTG at OD_{600nm}=0.6 in minimal medium containing 0.4% glucose/0.1% $^{15}NH_4Cl$ or 0.4% [$^{13}C_6$] glucose/0.1% $^{15}NH_4Cl$ in order to obtain ^{15}N- and ^{15}N/^{13}C-labeled proteins, respectively. The cell pellets were resuspended in ice-cold lysis buffer (6 M Guanidine HCl, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9, 5 mM imidazole), sonicated 4 × 30 s at full power, and centrifuged at 20 000 × g for 20 min at 4 °C. The supernatant was passed over a Ni^{2+}-agarose metal affinity column (Novagen, Inc), and the histidine-tagged protein was eluted according to manufacturer's intructions. The denatured dsRBD

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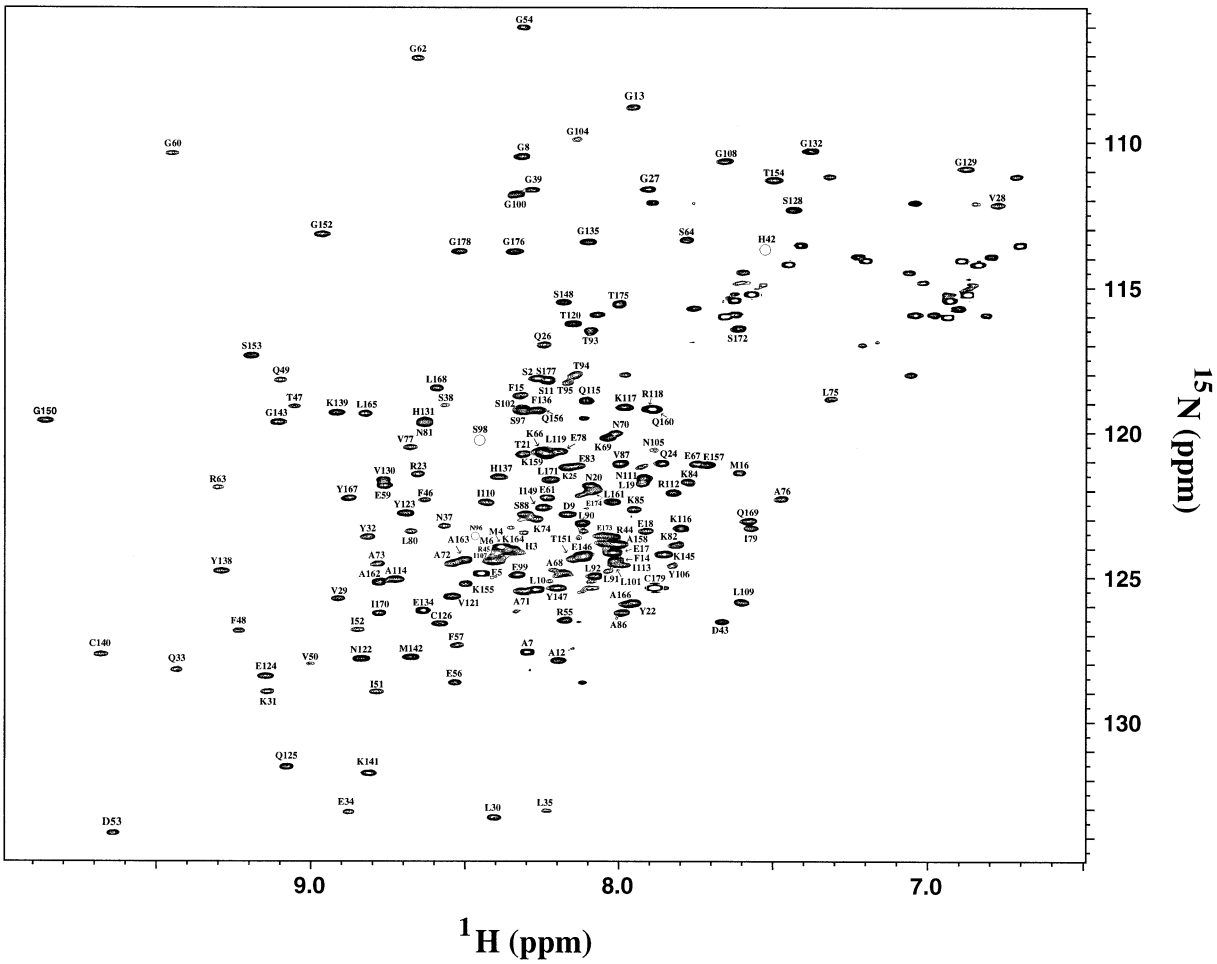

Figure 1. Two-dimensional ^{1}H–^{15}N HSQC spectrum of 0.9 mM uniformly ^{15}N-labeled dsRBD of human PKR at 25 °C pH 6.5, in 93% H_2O/7% D_2O with 20 mM sodium phosphate buffer and 1 mM DTT. The spectrum was acquired using a Varian Inova 500 MHz spectrometer with 1024 complex points in t_2 and 256 complex points in t_1 and processed by nmrPipe software. The assignments are labeled by the one-letter code of amino acids accompanied by a sequence number. H42, N96, and S98 were only observable at the lower contour and their positions are marked by circles.

was refolded by sequential dialysis into native buffer and the hisdine tag cleaved with commercial thrombin (Sigma, Inc). DsRBD was then further purified by gel filtration chromatography on a Superdex-75 column. The refolded dsRBD binds to dsRNA with the same affinity as wild-type PKR and was concentrated by an ultrafree-4 ultrafiltration device (Millipore, Inc). The dsRBD is mostly monomeric (>90%) as judged by gel-filtration chromatography, and its purity and concentration was determined by SDS-PAGE with Coomassie staining, UV spectrometry, and 23D ^{1}H–^{15}N HSQC experiment (Figure 1).

The dsRBD sample was prepared in argon-purged H_2O solution (7% $^{2}H_2O$), 100 mM NaCl, 20 mM sodium phosphate, pH 6.5, and 1 mM DTT in a 250 μl microcell NMR tube (Shigemi Inc., Allison Park, PA) at a concentration of approximately 0.9 mM. All the NMR experiments were conducted at 25 °C using a Varian Inova 500 MHz spectrometer equipped with a triple-resonance probe head and a shielded *z*-gradient unit. All of the NMR experiments are described in the reviews by Bax and Grzesiek (1993), Canavagh et al., (1995), and Kay (1995). The following experiments were recorded on a 0.9 mM uniformly $^{1}5N$-labeled protein. sensitivity-enhanced 2D ^{1}–^{15}N HSQC using water-flip-back for minimizing the water saturation, 3D water-flip-back ^{15}N-separated NOESY (mixing times 100 ms), 3D ^{15}N-separated HNHA. The

following experiments were recorded on a 0.9 mM uniformly labeled $^{15}N/^{13}C$-labeled protein: 2D-aro-$(H^{\beta})C^{\beta}(C^{\gamma}C^{\delta})H^{\delta}$, 2D-aro-$(H^{\beta})C^{\beta}(C^{\gamma}C^{\delta}C^{\epsilon})H^{\epsilon}$, 3D HNCACB, 3D CBCACONH; 3D (H)C(CO)NH, 3D H(CCO)NH, 3D HCCH-TOCSY, 3D $^{15}N/^{13}C$-edited NOESY, 4D HC(CO)NH TOCSY, 4D $^{15}N/^{13}C$-edited NOESY, and 4D $^{13}C/^{13}$-edited NOESY.

All the data were processed on Sun UltraSPARC workstation using nmrPipe Software (Delaglio et al., 1995). In the acquisition dimension, all data sets were processed identically. A solvent-suppression filter was applied to the time-domain data, followed by apodization with a 66° shifted squared-sine-bell window, zero-filling to the next power of 2, Fourier transformation, and phasing. The data were apodized in t_2 by a 72° shifted sine-bell window prior to zero-filling to 256 complex points, Fourier transformation, and phasing. For HNCACB, CBCACONH, HNHA, (H)C(CO)NH, and H(CCO)NH, the lengths of the ^{15}N time-domain data were doubled by mirror-image linear prediction, apodized by a squared cosine-bell window, zero-filled to 128 complex points, and Fourier transformed. The processed spectra were analyzed by the PIPP program (Garret et al., 1991; Garret et al., personal communication).

Extent of assignments and data deposition

Figure 1 shows the $^{1}H–^{15}N$ HSQC spectrum for uniformly ^{15}N-labeled 20 kDa dsRBD of PKR. Overall, the spectrum displays reasonably good chemical shift dispersion. However, dozens of composite cross peaks are present, which increased the difficulty during the course of the assignment. Except for the N-terminal 1–3 residues, F14, R45, A127, Q144, and Q160 which have missing NHs, all the non-proline residues (165 residues) have been unambigously assigned. More than 90% of the side-chains, including prolines and the aliphatic parts of the F14, A127, Q144, and Q160, have been assigned. The assignments have been deposited in the BioMagResBank database under number 4110. It should be noted that the N-terminal 1–5 and C-terminal 177–179 residues occur due to the cloning sites and do not belong to the natural sequence of PKR.

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